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SO-CALLED "REVERSED" HEMOLYSIS WITH FURTHER OBSERVATIONS ON THE MECHANISM OF HEMOLYSIS[1]

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An apparent reversal of hemolysis has been described by a number of observers. If real, such a phenomenon would be of great, and probably of general biological significance. And a study of the remarkable process by which a cell is, so to say, taken to pieces and then put together again, and a pigment with enormous molecules and constituting 30 per cent of the moist weight of the cell first extruded and then replaced, could not fail to throw light upon the physico-chemical structure of the erythrocyte and of other cells. The question has never been studied from this point of view and for the good reason that those observers who have most carefully investigated the phenomenon have convinced themselves that the reversal is only apparent. Others have observed these or related changes without studying them. Thus Wooldridge (1881) noticed that an apparently clear solution of laked corpuscles can be made to become more opaque by addition of free acid or acid sodium sulphate. The careful investigation of Peskind (1902) of the action of acids and acid salts in agglutinating and precipitating blood corpuscles and other cells is of interest in this connection.

Spiro (1898–99), in describing his technique for laking blood in order to determine its base and acid capacity, states that he used water saturated with ether for laking because when water alone was employed, "durch Zusatz von Salzen zu lackfarben gemachten Blut, resp. zu in Wasser aufgelösten rothen Blutkörperchen, wird nun aber der Farbstoff aus der wässerigen Lösung in die geschrümpften Scheiben hineingedrängt."

[1] A résumé of the work was given in Proceedings of the American Physiological Society (December, 1924), This Journal, 1925, lxxii, 238.

It was shown in 1901 by one of us that apparent reversal of hemolysis can be obtained when it is clearly demonstrable that no blood pigment has left the corpuscles and where indeed the condition of the pigment is such as to preclude its doing so. In these experiments mammalian corpuscles (Stewart, 1901; 1902 a), or still better the large erythrocytes of Necturus (Stewart, 1901; 1902 b) were fixed by 4 per cent formaldehyde to such a degree that actual exit of blood pigment does not occur on heating in a dilute solution of ammonia, although they swell greatly, become pale and appear to be laked. The suspending liquid contains no blood pigment whatever. Yet when now acted upon by hypertonic solutions the erythrocytes *appear* to re-accumulate the pigment. In the large erythrocytes the growing in of the yellow color is especially striking. It was shown clearly that the appearances are entirely due to the redistribution of the pigment within the corpuscles, the concentration diminishing when they swell and increasing when they shrink.

Since that time several writers have touched upon the subject, some holding that the hemoglobin actually reënters the ghosts or is accumulated on them under the influence of salt solutions of various concentrations and in other ways. It is perhaps scarcely worthy of remark today when so many writers seem to have adopted the convenient doctrine that it is not necessary to know the literature, that few, especially of the more recent workers on the so-called reversal of hemolysis, seem to know what has been done by their predecessors. Priority may, however, be granted to one recent suggestion, namely that the hemoglobin lies in the surface of the erythrocyte. Twenty-five years ago nobody was bold enough to imagine a "surface" layer which, if the pigment were dry, would constitute nearly a third of the volume of the cell and nearly two-thirds if water and pigment were in equal proportions.

Koeppe (1905) writes: "Noch nie ist beobachtet worden, dass lackfarbenes Blut wieder deckfarben wurde; das Lackfarbenwerden ist immer als Kriterium dafür betrachtet worden, dass die roten Blutscheiben zerstört-tot-seien. Der Vorgang des Lackfarbenwerdens ist als ein irreversibeler bezeichnet worden." He then goes on to state that the column of erythrocytes packed together in the hematocrit transmits the light like laked corpuscles, the diminished opacity being due to the pressing out of the suspending liquid so that the mass of erythrocytes in exact contact constitute but a single phase. When the sediment is redistributed it is seen that the individual erythrocytes retain their hemoglobin. Our own experience is that the column of erythrocytes in the hematocrit is not nearly so transparent as really laked blood. This scarcely needs proof. Anybody who cares to convince himself of it need only compare the sediment and the actually laked blood in tubes of larger diameter than the hematocrit tubes. Some of the suspending liquid is inevitably trapped

between the erythrocytes. It is possible that some of it may even enter the close packed corpuscles. In either case the volume of the sediment of corpuscles obtained by centrifugalization is liable to be greater than the true value. For instance the hematocrit usually gives a greater percentage volume of sediment than that deduced from the conductivities of the blood and serum (Stewart, 1899 b; 1924). Since in the electrical method the erythrocytes are floating free in a natural suspension their volume is accurately determined if the formula connecting the volume and the conductivities is properly constructed. This was done by making a series of dilutions of a sediment of dog's erythrocytes by adding accurately measured volumes or weights of the serum. The residual serum in the sediment was estimated colorimetrically. Any error in the colorimetric determination was of course rapidly reduced in the successive dilutions. The point has been missed by a recent writer (Gram, 1924), who says that "direct hematocrit measurement of cell volume 'in his study' is probably a more accurate method for cell volume than the colorimetric method. . . . used by Stewart." There is no reason in the nature of things why the hematocrit should be more accurate than a colorimetric method. But a preliminary question, on which no light is thrown in the excellent paper mentioned, is, How does one determine by the hematocrit the residual serum in a well centrifuged sediment of blood?

In the following experiment we compared the volume of corpuscles estimated by the electrical method, Hoppe-Seyler's method and the hematocrit. Rabbit's defibrinated blood was centrifuged for two hours at high speed. In the large tubes the sediment constituted 32 to 33 per cent of the volume. The values of $K\ (25°) \times 10^4$ were: for the blood 71.6; for the serum 125.6; and for the sediment 3.0. By the electrical method the corpuscles were calculated at 26.5 cc. in 100 cc. blood by formula (b), and at 26.1 cc in 100 cc. blood by formula (a) (Stewart, 1924). By Hoppe-Seyler's method the protein and hemoglobin in 100 grams blood were 15.896 grams; in the sediment of 100 grams blood 11.106 grams. The protein in 100 grams of serum was 6.524 grams. From these data the serum in 100 grams blood was calculated at 73.4 grams. The specific gravity of the blood was 1.0485, of the serum 1,0240, so that by volume the serum was 74.4 cc. in 100 cc. of blood, and the corpuscles 25.6 cc. The agreement with the electrical method is satisfactory. This shows incidentally that the formulae derived for dog's blood fit rabbit's blood also sufficiently well. The specific gravity of the erythrocytes was about 1.116.

The hematocrit gave a decidedly greater volume for the sediment even after very prolonged centrifuging at high speed. Thus in one trial the sediment was 63 per cent after 15 minutes, 40 per cent after 30 minutes more, 37 per cent after 30 minutes more, 33.5 per cent after 50 mimutes more, 33 per cent after 90 minutes more, and 32 to 31.5 per cent after 150 minutes more. In a second trial the corpuscle percentage was 37 after 55 minutes, 35 after 70 minutes more, 32 after 165 minutes more. After an additional rotation of 3 hours the sediment was slightly over 31 per cent, and after 3 hours more (making nearly 11 hours in all) it was slightly less than 31 per cent.

The hemoglobin of the blood was 64 per cent of Haldane's scale, that of the sediment from the centrifuge tubes 196 per cent (mean of five readings). The hemoglobin constituted somewhat less than 32 grams in 100 cc. erythrocytes, or about 28.5 per

cent of their weight. This is on the assumption that the erythrocytes make up 26.5 per cent by volume of the blood. The specific gravity of the hemoglobin would be about 1.3. If the erythrocytes were taken as constituting 32 per cent by volume of the blood the hemoglobin would be only 23.5 per cent of their weight, and the specific gravity of the hemoglobin would have to be at least 1.4 to conform with the measured specific gravities.

When a little saponin in substance was added to a sediment of the well centrifuged blood K (25°) $\times$ 10^4 rose from 3.0 to 62.8. The mere addition of the saponin would have produced hardly any effect upon the conductivity since the same amount added to serum diminished the conductivity by less than 3 per cent. The difference in the appearance of the really laked sediment and the unlaked sediment was great, the former being transparent, the latter opaque. Under the microscope it could be seen that in the unlaked sediment, although the erythrocytes were of course very numerous, currents could readily be set up, and they were not all closely in contact. It may be pointed out that it is impossible to pack disc-shaped bodies so as to obliterate completely the spaces between them without causing considerable distortion. Some of the erythrocytes were, as a matter of fact, facetted. The saponin-laked sediment was crowded with ghosts.

Matthes (1902) in connection with the fact discovered by him that dilute hydrochloric acid brings the pigment out of sublimate-fixed erythrocytes, mentions an experiment in which he supposes that on standing the ghosts may again take up the blood-pigment from the liquid. But Stewart and Peskind (1901; 1902 a, b) proved that when dogs' sublimate-fixed corpuscles, apparently laked when heated in water, appear to take up blood-pigment again on the addition of hydroxylamine hydrochlorate, the laking was only apparent, the corpuscles being swollen and the pigment, without leaving the erythrocytes, becoming less concentrated. Peskind (1902), while confirming the statement of Matthes that hydrochloric acid in a certain strength easily lakes sublimate-fixed mammalian corpuscles, which swell enormously, showed that if the swollen corpuscles are caught at a certain stage, at which they appear free from hemoglobin under the microscope and their suspension in water appears laked to the eye, they can be caused to shrink by hydroxylamine hydrochlorate, when their color returns and the opacity of the suspension is restored. At this stage it can be demonstrated that the swollen corpuscles still contain most of the blood pigment.

Rohonyi (1916) asserts that the reversal of hemolysis has been observed on the addition of protein-coagulating substances to hemolysed blood.

Adair, Barcroft and Bock (1921) dialyzed blood and placed a drop on a slide with saline solution. At once the preparation assumed the appearance of blood. The corpuscles retain a great part of their hemoglobin. They conclude that the solutions clearly consisted of a mass of corpuscles which, as dialysis proceeded, gradually swelled up until they finally almost lost their refrangibility. Consequently it became possible to see light through the solution.

Recently Brinkman and Szent-Györgyi (1923–4) stated that it is easily demonstrable that in blood laked by a proper concentration of linolenic acid, the stromata are all present and not much swollen. If now a small amount of isotonic saline is added to this completely hemolysed blood, the hemoglobin is again taken up by the stromata. While their acquaintance with the literature seems meagre they are perfectly correct in their statement that the stromata are not destroyed. Many years ago it was pointed out and has since been repeatedly emphasized by one of us that in numerous forms of laking the stromata persist. On the other hand we believe Brinkman and Szent-Györgyi have misinterpreted their observations when they conclude that the hemoglobin which has left the erythrocytes returns to them under the influence of the procedures employed by them. The so-called reversal of hemolysis is, we think, only apparent. Bayliss (1924) has criticized the main conclusion of these authors. Rockwood (1924), by means of the ultramicroscope claims to have confirmed the view that hemoglobin actually returns to the erythrocytes, laked by distilled water, when hypertonic solutions (5 per cent KCl or NaCl, etc.) are added. We do not think that such observations as he has recorded permit a definite conclusion to be drawn. We are however in entire agreement with him when he states that he finds that so-called "amboceptor-complement" hemolysis is one of the milder forms of laking. This was pointed out by one of us many years ago and repeatedly since. The assumption often made that the stromata are destroyed is simply due to lack of proper search for them, (Baumgarten (1908) confirming Stewart (1899 a, 1901)).

Both in the previous work and in the present research "pseudo-reversal" has been investigated not as a separate problem but as a phenomenon which when related with other observations throws light upon the mechanism of hemolysis. Three questions bearing directly upon "reversion" have been asked: 1, Is hemoglobin present within the ghosts before the reversion phenomenon is brought about? The answer to this question is that in all the cases studied it has been shown that the ghosts already contain hemoglobin wherever the reversion reaction is obtainable. 2, Is the amount of hemoglobin already in the ghosts sufficient to produce the appearance of re-pigmentation? The answer is that it is sufficient. When these two questions have been answered in this way it would scarcely seem necessary to put the third one: 3, Is there any evidence that in reversion hemoglobin enters or is attached to the ghosts from the outside? The answer is that there is no good evidence that such is the case.

The observations fall into two main groups: *a*, mainly microscopic; *b*, observations mostly of a quantitative nature in which from conductivity determinations, hemoglobin estimations, etc., conclusions were reached as to some of the redistributions of water and solids occurring in hemolysis.

Microscopic observations. We have repeated the observations on formaldehyde-fixed erythrocytes (mammalian and also the large erythrocytes of Cryptobranchus). The results were precisely the same as before. Crystallization of hemoglobin either outside or inside the erythrocytes was not observed under the influence of laking reagents (on the unfixed erythrocytes of Cryptobranchus) although so remarkable a phenomenon in the Necturus corpuscles. The following table shows the dimensions of 10 of the formaldehyde-fixed corpuscles while swollen by the action of ammoniacal water and after treatment with Na_2SO_4 ($\frac{1}{2}$ M), which caused apparent reversal, as did also the addition of 1.6 to 4 per cent formaldehyde and 0.1 per cent $HgCl_2$. The erythrocytes, which had become quite pale under the influence of the ammoniacal water, without losing any blood-pigment to the suspending liquid, became colored again and looked like normal erythrocytes. The longitudinal and transverse diameters are simply given in terms of the scale of the eye-piece micrometer.

SWOLLEN CORPUSCLES	AFTER SHRINKING
2.6 × 1.4	1.4 × 0.9
2.7 × 1.5	1.8 × 0.9
2.2 × 1.2	1.6 × 0.8
2.5 × 1.1	1.6 × 1.0
2.7 × 1.4	1.6 × 0.9
2.7 × 1.4	1.5 × 1.0
2.2 × 1.4	1.6 × 0.8
2.3 × 1.5	1.5 × 0.9
2.4 × 1.4	1.4 × 0.6
2.6 × 1.4	1.6 × 0.8
Average 2.5 × 1.4	1.6 × 0.9

The diameters in the swollen and shrunken condition are approximately as 3:2. This is true of both the long and the short diameters. The shape of the corpuscle is therefore not essentially altered. The volume of the swollen corpuscles is increased more than threefold. The paleness of the corpuscles due to the diminished concentration of the blood pigment is thus easily explained.

The formaldehyde-fixed corpuscles were tested from time to time during two months for the form of pseudo-reversal described. In all this time they were kept in the formaldehyde and never failed to yield excellent reversion. Many observations were made with sodium oleate, saponin, water and other forms of hemolysis on fresh Cryptobranchus blood and washed erythrocytes, obtained from 6 specimens of Cryptobranchus, the longest a female 56 cm. long. In one experiment the conductivities of the blood and serum were measured. For the blood K (25°) × 10^4 was 56.3 and for the serum 121.2. The volume of erythrocytes calculated from the

conductivities was 36.3 per cent. The hematocrit gave the following readings: 10 minutes, 42 per cent; 20 minutes, 39; 30 minutes, 38.5; 40 minutes, 38; 50 minutes, 38 per cent. The agreement is excellent showing that even for corpuscles so different in size and shape from the dog's erythrocytes, for which the formulae were derived, there is no great discrepancy.

It may be mentioned that while rabbit's erythrocytes were hemolyzed by Cryptobranchus serum, rabbit's serum did not lake Cryptobranchus erythrocytes.

With unfixed Cryptobranchus erythrocytes it was seen that after laking in various ways and then adding certain solutions the nuclei became strongly tinged with the hemoglobin. What seems to happen is that when the nuclei are swollen in the laking process they become permeable for the hemoglobin as well as for water, possibly because the great stretching of the nuclear membrane leaves spaces through which the large hemoglobin molecules can pass. Then when the nucleus shrinks the hemoglobin or a portion of it is trapped within the nuclear membrane. Since the difference in the dimensions of the swollen and shrunken nucleus is very great the concentration of hemoglobin in the shrunken condition is so great that the nuclei appear deeply pigmented. The cytoplasm may at the same time appear colorless or tinged yellow, depending upon the degree of shrinking of the corpuscle. That there is nothing specific in the taking up of hemoglobin by the nucleus (which can also be observed in frog's erythrocytes with certain methods of treatment) is shown by the fact that leucocytes, both those of Cryptobranchus and of the frog, can be caused to become deeply tinged with it. Also epithelial cells (scrapings from the mouth of Cryptobranchus) were seen to become distinctly yellow when placed in the hemoglobin containing liquid of laked blood (either Cryptobranchus or mammalian blood), to which 1.6 to 4 per cent formaldehyde was then added. The reaction was rendered more rapid if saponin (a drop or two of 0.1 per cent solution) was added to a drop of the suspension of scrapings in laked blood before addition of formaldehyde. One or two illustrative protocols may be given.

October 28, 1924. Three cubic centimeters of rabbit's defibrinated blood were laked by 0.1 cc. of 2 per cent sodium oleate. Microscopically laking was complete. The addition of 5 per cent NaCl solution to a drop of the laked blood on a slide gave a good reaction, viz., shrinking, clumping and a greater concentration of color in the ghosts. When distilled water was now added the shrunken colored stromata again swelled and became almost invisible. A second addition of NaCl solution again caused reversion. The addition of Na_2SO_4 solution to the laked blood on a slide also gave good reversion. Ammonium chloride solution produced what looked like very fine corpuscles from the pale swollen ghosts. These, however, soon became pale again in the NH_4Cl solution, since this salt readily penetrates the corpuscles, but could be again "reversed" by 5 per cent NaCl solution.

January 29, 1925. Frog's erythrocytes.

SUSPENDED IN	LAKED BY	"REVERSION" BY	REMARKS
0.85% NaCl	Heat	0.1% $HgCl_2$	Some nuclei slightly colored
0.85% NaCl	Heat	½ mol. Na_2SO_4	Nuclei colored (?)
0.85% NaCl	Heat	4% formalin	Nuclei decidedly colored
0.85% NaCl	Heat	0.85% NaCl	Nuclei decidedly colored
0.85% NaCl	Heat	¼ mol. Na_2SO_4	Nuclei and leucocytes colored
0.85% NaCl	Heat	0.05% $HgCl_2$	Nuclei not colored
7.5% sucrose	Heat*	0.1% $HgCl_2$	Some nuclei colored
7.5% sucrose	Heat*	4% formalin	Nuclei decidedly colored
7.5% sucrose	Heat*	¼ mol. Na_2SO_4	Nuclei not colored
7.5% sucrose	Heat†	0.1% $HgCl_2$	Nuclei not colored
7.5% sucrose	Heat†	4% formalin	Nuclei not colored
7.5% sucrose	Heat†	0.85% NaCl	Nuclei not colored
0.85% NaCl	Dist. H_2O	4% formalin	Nuclei colored
0.85% NaCl	Dist. H_2O	0.1% $HgCl_2$	Nuclei not colored
0.85% NaCl	Dist. H_2O	½ mol. Na_2SO_4	Nuclei not colored
7.5% sucrose	Dist. H_2O	4% formalin	Nuclei apparently colored
0.85% NaCl	Na oleate	4% formalin	Nuclei not colored
0.85% NaCl	Na oleate	0.1% $HgCl_2$	Nuclei not colored
0.85% NaCl	Na oleate	¼ mol. Na_2SO_4	Nuclei not colored
0.85% NaCl	0.5% saponin	4% formalin	Nuclei colored
0.85% NaCl	0.5% saponin	0.1% $HgCl_2$	Nuclei colored
0.85% NaCl	0.5% saponin	¼ mol. Na_2SO_4	Nuclei colored (?)

* Laking not quite complete.

† Laking complete.

The corpuscles left till next day in salt solution and sugar solution no longer show the "reversion" and the taking up of hemoglobin by the nuclei as well as they did, whereas those left in serum still give a good reaction.

Coloring of the nuclei of the erythrocytes was also seen when a formaldehyde solution was added to laked blood from a frog, into the dorsal lymph sac of which dog's bile had been injected on the previous day.

"Reversion" was also studied microscopically in mammalian blood laked by freezing and thawing and in blood laked spontaneously by standing for a long time. No essential difference was seen in the appearances from those studied with other forms of laking. That the reaction is not associated with the "life" of the erythrocytes is already clear enough, but it is perhaps most strikingly demonstrated when reversion is produced in blood laked spontaneously when no precautions have been taken to exclude bacteria.

Not only the state of vitality of the erythrocytes but the nature of the suspending liquid can be widely varied without abolishing the reaction. Thus rabbit's corpuscles in 10 per cent cane sugar solution laked by saponin or by heat give reversion with many or all of the reagents which give positive results in salt solution or serum suspensions.

While we have not made any systematic comparison of the reaction in different kinds of mammalian blood it seemed that pig's erythrocytes gave better reversion (both macroscopically and microscopically) than any of the other kinds of blood used. On the other hand it was not found nearly as suitable as rabbit's blood for the quantitative experiments on conductivity, etc., as the ghosts seemed to be far more difficult to separate by the centrifuge.

Perhaps the most direct indications that in the "reversal" phenomenon the hemoglobin which appears in the stromata is not taken up from the outside but is already within them, are the following:

Clumps composed of numerous ghosts closely in contact with each other can often be found with various forms of laking. When these are watched under the microscope and a solution which causes "reversion" allowed to to act on them the apparent return of pigment is far more striking in the larger clumps than in smaller clumps or isolated ghosts. Now the latter in a solution of hemoglobin would have a much better chance of taking up the pigment from the outside and when then clumped ought to show more of it. In clumps of ghosts filling practically all the space between the cover slip and slide and closely in contact with each other there cannot be much of the hemoglobin solution in contact with the erythrocytes. The pigment which they quickly seem to take up is therefore probably already within them. It is shown quantitatively in numerous experiments in the following pages that the ghosts do contain hemoglobin *before* "reversion" and in about the same concentration as the suspending liquid of laked blood or laked erythrocyte suspensions. A whitish appearance of a sediment is not a proof that it does not contain as much hemoglobin as the suspending liquid. For it may be due simply to greater scattering of the light. Only a hemoglobin estimation can decide.

Ghosts freed from most or all of the hemoglobin-containing suspending liquid may still show the reversion phenomenon on the addition of suitable reagents. Here the hemoglobin cannot have come from the outside.

When blood or a suspension of erythrocytes was laked and reversion then caused and the "reversed" ghosts centrifuged off, no evidence was obtained from hemoglobin estimations that the suspending liquid had lost the hemoglobin which it must have lost had the pigment apparently accumulated in the ghosts come from the outside.

That the apparent reversion is not a replacing of the escaped hemoglobin in its natural condition in the stromata is indicated by other simple experiments. For example, frog's erythrocytes laked by heat and in other ways were centrifuged off and washed once with physiological saline to get rid of the hemoglobin-containing suspending liquid. The laked frog's ery-

throcytes were then placed on a slide in the hemoglobin-containing liquid from rabbit's laked corpuscles, after separation of a large number of the rabbit's ghosts. By addition of mercuric chloride and other solutions the frog's ghosts can be made apparently to take up hemoglobin and become quite as deeply colored as the "reversed" rabbit's ghosts. If the "restored" frog's corpuscles contain frog's hemoglobin it is clearly not taken up from the outside in this experiment; if they contain rabbit's hemoglobin they are abnormal frog's erythrocytes. That the change is quite artificial is indicated by the fact that with laked frog's erythrocytes various solutions caused the nuclei to become decidedly yellow.

Rabbit's washed ghosts placed in the supernatant liquid of rabbit's laked erythrocytes may appear to take on color when acted on by one or other of the solutions which shrink them. When centrifugalized, however, and washed, sometimes even once, with 0.85 per cent NaCl solution they give up the pigment, if any, which they have taken up. Their resemblance to normal erythrocytes when "reversed" under the microscope is therefore only apparent, for normal erythrocytes are not washed free from pigment in this way.

The large number of solutions of quite different nature which will cause reversion (better or worse according to the form of laking and other circumstances) indicates that the change is in no sense a rebuilding of the erythrocyte. For instance, ghosts of heat-laked and of water-laked rabbit's erythrocytes were reversed in one experiment by 0.85 per cent and by 2 per cent NaCl solution and by formaldehyde solutions up to 4 per cent. In another experiment rabbit's washed corpuscles were laked with a minimal amount of sodium oleate. Very distinct clumping and reversal were caused by 5 per cent, 2 per cent and 0.85 per cent NaCl solution, smaller effects by 1 per cent formaldehyde and by 0.1 per cent mercuric chloride. In other observations osmic acid was seen to cause good reactions. Sodium sulphate solutions up to $\frac{1}{2}$ M strength was one of the most effective agents.

In connection with the laking of nucleated erythrocytes it may be pointed out that the suspending liquid should have a higher concentration of hemoglobin than the laked blood if the nuclei have not taken up hemoglobin, even if the extranuclear stroma has about the same hemoglobin concentration as the suspending liquid. From the difference the volume of the nuclei with different methods of laking might perhaps be calculated approximately.

Quantitative experiments on conductivity, hemoglobin concentration, etc., of unlaked and laked blood and suspending liquids. This constituted the main portion of the research. The experiments were planned to yield data on the total volume of ghosts as compared with that of erythrocytes in various forms of laking, the concentration of hemoglobin in ghosts and suspending

liquids, the number of ghosts as compared with the number of erythrocytes, etc.

In rabbit's blood and sheep's blood (either the defibrinated blood or suspensions of washed erythrocytes in salt solution, or both) we have determined: *a*, the electrical conductivity of the blood or suspension, of the serum or suspending liquid, and of the sediment before and after laking with sodium oleate, heat, saponin or immune serum; *b*, the hemoglobin concentration in the same fractions. These observations enable us to estimate the proportion of the total volume occupied by erythrocytes before laking and by ghosts after laking (hematocrit readings were also made), and what proportion of the total hemoglobin is still contained in the ghosts after laking. It will be seen that the volume of the ghosts with careful laking was always quite substantial, not infrequently as great as that of the unlaked corpuscles, and that the concentration of hemoglobin in the laked suspension, the suspending liquid and the sediment of ghosts was practically the same. It follows that the ghosts contain a large amount of hemoglobin (the simplest assumption is that they are soaked with the same hemoglobin solution in which they float), and that when they are now acted upon by solutions of salts, etc., it is the hemoglobin already in their interior which causes them again to appear pigmented. That the chief part of the contents of the ghosts cannot differ much from the suspending liquid was confirmed by specific gravity determinations on the laked blood or suspension, the sediment of ghosts and the suspending liquid freed from ghosts. The difference in the specific gravity of these fractions was very small.

Some typical experiments on each of the four forms of laking mentioned will now be given.

Sodium oleate laking. November 24, 1924. Obtained 150 cc. of blood from two rabbits; mixed and defibrinated. Some of the blood was washed with 0.85 per cent salt solution and the sediment made up approximately to the original volume of the blood. The blood and suspension were laked by addition of 2 per cent sodium oleate solution in distilled water. The aim was to use the minimum quantity which would cause complete laking. The blood and suspension were kept at 37°C. after addition of the oleate till laking was complete. More oleate was, of course, required for the blood than for the suspension of washed corpuscles.

Conductivity measurements. For the defibrinated blood K (25°) $\times$ 10^4 was 59.9; for the serum 126.2; for the defibrinated blood laked by oleate 51.1; for the suspending liquid of the laked blood freed from ghosts 91.5. The liquid was always freed from ghosts by prolonged centrifugalization at a high speed. Tests were made from time to time with the microscope before and after the addition on the slide of a solution previously shown to cause good reversal and clumping of the ghosts.

The relative volume of corpuscles in the blood determined from the conductivities was 34.9 per cent, and the volume of ghosts in the laked blood 27.4 per cent. With the hematocrit the following readings were obtained for the sediment of the unlaked blood: (10 minutes) 42 per cent, (20 minutes) 41 per cent, (30 minutes) 40.5 per cent, (40 minutes) 40 per cent, (50 minutes) 39.5 per cent, (70 minutes) 39.5 per cent.

The hemoglobin in the defibrinated blood corresponded to 72 per cent of the scale of Haldane's hemoglobinometer; in the laked defibrinated blood (which had been diluted by the oleate solution) 56 per cent. The liquid of the laked blood freed from ghosts had a concentration of hemoglobin estimated at 62 per cent of the scale; the sediment of the laked blood 55 per cent.

The specific gravity of the defibrinated blood was 1.052; of the serum, 1.027; of the erythrocytes (calculated) 1.096; of the liquid from the laked blood freed from ghosts 1.038; of the sediment of ghosts, 1.041. The specific gravity was determined by the pycnometer.

For the suspension of washed corpuscles (unlaked) K (25°) $\times$ 10^4 was 76.0; for the liquid from this suspension 150.2; for the laked suspension 55.7; for the liquid separated from the laked suspension 110.1. The erythrocytes made up 32.0 per cent of the volume of the suspension and the ghosts 32.7 per cent of the laked suspension, as calculated from the conductivities.

It may be pointed out that from the theory of the electrical method it follows that the calculated volume of the ghosts represents the minimum volume which can be present. If the permeability of the ghosts were greater than that of the erythrocytes and their electrical resistance less, the effect upon the calculated relative volume of ghosts would be to make it less than the true volume. From such measurements as have been made there is no indication that the specific electrical resistance of the ghosts is sensibly diminished as compared with that of the erythrocytes, at least when the ghosts are still capable of being shrunk by hypertonic solutions and of showing the reaction of "pseudo-reversal."

The suspension of washed corpuscles had a hemoglobin concentration corresponding to 71 per cent of Haldane's standard, the laked suspension (diluted by the oleate solution) a concentration corresponding to 60 per cent, the suspending liquid of the laked suspension freed from ghosts, 57 per cent, and the sediment from the laked suspension, 59 per cent.

For the oleate solution K (25°) $\times$ 10^4 was 19.1; for serum from the defibrinated blood *plus* oleate solution in the same proportion in which it was added to the defibrinated blood it was 107.7. For the 0.85 per cent NaCl solution K (25°) $\times$ 10^4 was 153.0, and for the salt solution *plus* oleate solution in the same proportion in which it was added to the suspension of washed corpuscles it was 140.0.

From a consideration of the relative total volume of ghosts and erythrocytes, the conductivities and hemoglobin percentages of the various fractions certain conclusions can be reached as to changes occurring in the hemolytic process. Let us take, for instance, the suspension of washed erythrocytes in the experiment just described. Allowing for the dilution of the suspension by the oleate solution it would contain immediately after admixture not 32 per cent but about 27 per cent of erythrocytes, with a total amount of hemoglobin of about 29 per cent of their moist weight, or 8 grams in the erythrocytes of 100 cc. of suspension. In laking about 5.7 grams passed into the suspending liquid, leaving 2.3 grams in the ghosts.

Taking account of the difference between the specific gravity of hemoglobin and unity this would make the ghosts only 22.5 cc. in 100 cc. of suspension. Since they actually constituted 32.3 per cent of the laked suspension they must have taken up about 10 cc. of water, namely, 4.5

cc. in return for the hemoglobin given off and an additional 5.3 cc. from the suspending liquid. If they contained 18 cc. of water before laking they must have contained fully 50 per cent more after laking. Here any possible exchange of other constituents than hemoglobin and water has been neglected.

The suspending liquid was diluted approximately 20 per cent by the oleate solution and 6 per cent by hemoglobin. Deducting from the 26 per cent, 14 per cent representing the water which passed into the erythrocytes, we get a net dilution of about 12 per cent. This would diminish the conductivity of the suspending liquid of the unlaked suspension from 150 to about 131, taking no account of changes in ionisation. The depressing action of hemoglobin as a non-electrolyte would cause a further diminution, certainly to about 115, possibly somewhat more. This brings us very near to the conductivity actually observed for the suspending liquid of the laked suspension, viz., 110. It would appear then that the laking process was not accompanied by any significant loss or gain of electrolytes by the erythrocytes.

Another experiment with oleate laking may be cited as an illustration of hemolysis carried to the point at which the volume of ghosts, although still substantial, is decidedly less than the original volume of erythrocytes.

December 3, 1924. Obtained about 80 cc. of defibrinated blood from two rabbits and mixed. Washed with 0.85 per cent NaCl solution and made up to original volume. Laked 70 cc. of the suspension with 2.3 cc. of 2 per cent sodium oleate solution in distilled water at 40°C. in 10 minutes. Pseudo-reversal was obtained. On centrifugalisation the supernatant liquid showed no ghosts even after addition of solutions which cause pseudo-reversal.

Preliminary observations were made on a small sample of the suspension to determine the minimum amount of oleate necessary for complete laking. For the unlaked suspension $K\ (25°) \times 10^4$ was 78.7; for the laked suspension 68.8; for the suspending liquid separated from the unlaked suspension 151.2. These results gave 31.9 per cent of erythrocytes in the suspension, and the volume of ghosts after laking was little less. However, in the main experiment, when a proportional amount of the oleate was added, for some undiscovered reason (possibly because the suspension had stood somewhat longer) laking proceeded more rapidly than had been expected, and also farther, as indicated by the fact that the conductivity of the laked suspension in the main experiment was somewhat greater than that of the unlaked suspension, whereas in the preliminary experiment it was decidedly less. $K\ (25°) \times 10^4$ was 83.0 for the laked suspension, and 118.0 for the liquid freed from ghosts, giving 17.1 per cent of ghosts. The hematocrit gave the following readings for the sediment from the laked suspension: after 10 minutes, 25 per cent; 20 minutes, 21 per cent; 30 minutes, 20 per cent; 40 minutes, 20 per cent.

The hemoglobin in the unlaked suspension was 70 per cent of Haldane's scale, in the laked suspension freed from ghosts 68 per cent, in the sediment from the laked suspension 66 per cent.

The specific gravities were as follows: unlaked suspension, 1.0379; suspending liquid of unlaked suspension, 1.0085; laked suspension, 1.0377; liquid from laked suspension freed from ghosts, 1.0371; sediment from laked suspension, 1.0400.

The hemoglobin in 31 cc. of erythrocytes was about 9 grams. In laking about 7.5 grams passed into the suspending liquid, leaving about 1.5 gram in 17 cc. of ghosts. In addition to 6 cc. of hemoglobin, about 8 cc. of water must have left the 31 cc. of erythrocytes on balance. The effect of the small quantity of oleate solution in diluting the suspending liquid is neglected for simplicity.

The conductivity of the suspending liquid of the unlaked suspension would be reduced by dilution with water and hemoglobin from 151 to about 125. The depressing effect of the hemoglobin as a non-conductor would easily account for the difference between this and the conductivity of the suspending liquid of the laked suspension, so that here also there is no need to assume that any material exchange of electrolytes occurred. If anything, there may have been a small loss of electrolytes, on balance, by the erythrocytes.

In another experiment (Nov. 3, 1924), of which the details will be omitted, 25 cc. of rabbit's defibrinated blood were laked by 7.5 cc. of a 2 per cent sodium oleate solution in water at 37°C. and 30 cc. of a suspension of washed corpuscles by 3 cc. of the oleate solution. The values of K (25°) $\times$ 10^4 were: defibrinated blood 66.7; laked defibrinated blood 48.2; serum from defibrinated blood 127.5; liquid from laked defibrinated blood 90.4; suspension of washed corpuscles 81.9; laked suspension 60.8; suspending liquid from unlaked suspension 154.0; liquid from laked suspension 115.0; sediment of the laked suspension 19.5. The erythrocytes constituted 30.6 per cent of the defibrinated blood (34 per cent by hematocrit after 30 minutes' rotation), and the ghosts 29.8 per cent of the laked blood. On standing overnight in the refrigerator the proportion of ghosts in the laked blood diminished to 13.1 per cent. The erythrocytes constituted 29.5 per cent of the suspension of washed corpuscles, and the ghosts 30.9 per cent of the laked suspension, (hematocrit 35 per cent after 10 minutes, 32.5 per cent after 20 minutes, 31 per cent after 30 minutes, 30 per cent after 40 minutes, 29.5 per cent after 50 minutes). On standing overnight in the refrigerator the laked suspension showed only 23.4 per cent of ghosts. The hemoglobin in the 30.6 cc. of erythrocytes in the defibrinated blood was about 7.5 grams.

Heat laking. December 5, 1924. A suspension of washed rabbits' erythrocytes was laked by careful heating between 56° and 60°C for 30 minutes. Good pseudo-reversal obtained with $\frac{1}{2}$ M Na_2SO_4. The values obtained for K (25°) $\times$ 10^4 were: the unlaked suspension 80.2; the suspending liquid 150.7; the laked suspension 72.9; the liquid from the laked suspension freed from ghosts 126.9 The erythrocytes constituted 29.8 per cent of the unlaked suspension and the ghosts 26.1 per cent of the laked suspension, calculated from the conductivities. The hemoglobin in the laked suspension gave 65 per cent of Haldane's scale, and in the liquid freed from ghosts 60 per cent. The specific gravity of the unlaked suspension was 1.0378; of the suspending liquid separated from it 1.0083; and of the liquid separated from the laked

suspension 1.0352. As evaporation was prevented during heating, the specific gravity of the laked suspension would of course be the same as that of the unlaked.

The percentage of suspending liquid after laking was little greater than before laking. Taking the hemoglobin in the suspending liquid of the laked suspension at 8 per cent by weight, about 6 grams of hemoglobin must have passed out of 30 cc. of erythrocytes, which contained originally about 8 grams. About 5 cc. of water must have entered the erythrocytes to replace this hemoglobin, had the suspending liquid been just of the same volume as that of the unlaked suspension. It is not, however, precisely of the same volume but about 4 cc. more in 100 cc. of suspension, so that on balance only 1 cc. of water has gone into the 30 cc. of erythrocytes. The 2 grams of hemoglobin in the 26 cc. of ghosts therefore exist after laking in a concentration of 7.7 per cent, which agrees well with the colorimetric determination.

The net effect of the exchange of water and hemoglobin would be to dilute the erythrocytes in the suspending liquid about 5.7 per cent, reducing the conductivity of the suspending liquid of the unlaked suspension from 150.7 to about 142. The depressing effect of hemoglobin as a nonconductor on the conductivity can be taken as responsible for a further reduction to practically the conductivity of the suspending liquid of the laked suspension. On balance, therefore, electrolytes have neither passed into nor out of the erythrocytes during heat laking.

Saponin laking. December 9, 1924. Defibrinated rabbit's blood was washed once with 0.85 per cent salt solution and made up to the original volume with the salt solution. Seventy cubic centimeters of the suspension were laked with 1.5 cc. of a 0.5 per cent solution of saponin in 0.85 per cent NaCl solution at 37°C. in 15 minutes. "Reversal" was good. We have had no difficulty in obtaining reversal with saponin; although Bayliss (1924) says he could not get it. Naturally the dose must be considered.

The values for K (25°) $\times$ 10^4 were: the suspension (unlaked), 79.0; the liquid separated from it, 149.3; the laked suspension, 84.4; the liquid from the laked suspension freed from ghosts, 122.4; the defibrinated blood, 70.0; the serum, 126.9; the 0.85 per cent NaCl solution, 149.3; the 0.85 per cent NaCl solution plus saponin solution in the same proportion as added to the suspension, 149.7; the saponin solution, 151.1. The corpuscles made up 28.0 per cent of the blood and 30.9 per cent of the suspension of washed corpuscles; the ghosts made up 17.8 per cent of the laked suspension, as calculated from the conductivities. The hematocrit readings for the sediment in the laked suspension were 27.5 (10 minutes), 24 (20 minutes), 23 (30 minutes), 21.5 (40 minutes), 21 (50 minutes), 20.5 (70 minutes), 20 (80 minutes).

The hemoglobin in the laked suspension was 67 per cent of Haldane's scale, in the sediment 65 per cent, in the liquid freed from ghosts 68 per cent.

The specific gravity was 1.0486 for the blood, 1.0262 for the serum, 1.0357 for the suspension of washed corpuscles, 1.0067 for the suspending liquid separated from the washed corpuscles, 1.0355 for the laked suspension, 1.0350 for the liquid freed from ghosts of the laked suspension, 1.0377 for the sediment from the laked suspension.

The hemoglobin in the 30.1 cc. of erythrocytes in 102 cc. of the laked suspension (allowing for the addition of the saponin solution) was about 8.9 grams. Of this about 7.3 grams passed into the 72 cc. of suspending liquid and 1.6 grams remained in the 18 cc. of ghosts. In addition to about 6 cc. of hemoglobin about 6 cc. of water must have passed from the erythrocytes into the suspending liquid. The specific conductivity of the suspending liquid would not be altered by the addition of the saponin solution. Dilution of the suspending liquid with 12 cc. of water and hemoglobin would reduce the conductivity from 149 to about 128, and the depressing influence of the hemoglobin would reduce it well below the observed value. From this it follows that electrolytes as well as water must have come out of the erythrocytes on balance.

Here the ghosts made up somewhat more than half the volume occupied by the unlaked erythrocytes instead of approximately an equal volume (or even more), as was sometimes seen with the milder laking agents. To see whether the proportion of ghosts would be favorably influenced by increasing the proportion of erythrocytes in the suspension, another experiment was made.

December 12, 1924. Defibrinated rabbit's blood was washed twice with 0.85 per cent NaCl solution and then made up to half its original volume with the salt solution. As usual, the blood was freshly drawn. Preliminary tests were made to determine the minimum laking dose, which was found to be 0.1 cc. of 0.1 per cent saponin solution (in 0.85 per cent NaCl) for 1 cc. of the suspension. Then 35 cc. of the suspension was laked with 3.5 cc. of the saponin solution. Good reversion was obtained under the microscope.

The values of K (25°) $\times 10^4$ were: the suspension (unlaked), 45.8; the suspending liquid separated from it, 149.3; the laked suspension, 60.4; the liquid separated from the laked suspension and free from ghosts, 108.8; the sediment from the laked suspension (jelly-like), 38.3. It was calculated that the erythrocytes constituted 54.4 per cent of the suspension and the ghosts 27.7 per cent of the laked suspension.

The hemoglobin in the laked suspension was 95 per cent of Haldane's scale; in the liquid free from ghosts 93 per cent, and in the sediment 95 per cent.

Allowing for the dilution with the saponin solution, 100 cc. of the unlaked suspension at the moment after addition of the saponin would contain 49.5 cc. of erythrocytes with about 12.5 grams of hemoglobin. In laking, about 9 grams of hemoglobin must have passed out into the suspending liquid, leaving 3.5 grams in 28 cc. of ghosts. But the volume of the suspending liquid in 100 cc. of the suspension was increased by 21.7 cc., so that in addition to 7 cc. of hemoglobin nearly 15 cc. of water must have left the erythrocytes. The dilution alone, apart from any change in the dissociation, would reduce the conductivity of the suspending liquid from its value before laking to or below its value after laking. If to this be added the reduction due to the depressing influence of hemoglobin, the conductivity of the suspending liquid of the laked suspension would go

down so far below the actual conductivity as to make it necessary to assume that the suspending liquid must have gained electrolytes as well as water and hemoglobin from the erythrocytes.

Laking by specific antibody. Rabbits were immunized in the usual way with washed sheep's erythrocytes and when the antibody titer had reached approximately the maximum, four experiments were made on the same plan as those described for the other forms of laking.

February 27, 1925. Sheep's blood was washed twice and made up to the original volume with 0.85 per cent NaCl solution. Twenty-eight cubic centimeters of the suspension were laked by addition of 4 cc. of rabbit's immune serum, and 8 cc. guinea pig's serum, kept at 37°C. for 1 hour. Laking was then complete and pseudo-reversion good. The quantities of serum added were large because it was desired to use a suspension containing a larger proportion of erythrocytes than is generally employed in serology, so as to be able to estimate the volume of ghosts more accurately.

The values of K (25°) $\times 10^4$ were: for the unlaked suspension, 74.4; liquid separated from the suspension, 151.2; laked suspension, 89.0; the liquid free from ghosts separated from the laked suspension, 119.4; the unlaked suspension *plus* rabbit's serum heated to inactivate it and heated guinea pig's serum (both sera in the same proportion as used for laking), 84.4; and the liquid separated from this suspension, 144.0.

It was calculated from the conductivities that the volume of erythrocytes in the unlaked suspension (before addition of the rabbit's and guinea pig's serum) was 31.6 per cent, and of ghosts in the laked suspension 14.8 per cent. The final hematocrit reading for the unlaked suspension gave 33 per cent of erythrocytes. The volume of erythrocytes calculated for the suspension with heated serum added was 25.1 per cent, the volume of erythrocytes calculated from that known to exist in the unlaked suspension and the known volumes of serum added on the assumption that all the added serum went to augment the volume of the suspending liquid was 22.1 per cent.

The concentration of hemoglobin in the laked suspension was 48 per cent of Haldane's scale; in the suspending liquid free from ghosts 50 per cent; in the sediment 50 per cent.

The specific gravities were: the suspension of corpuscles in 0.85 per cent NaCl solution, 1.0403; suspension after addition of the rabbit's and guinea pig's serum, 1.0361; liquid separated from the laked suspension, 1.0299; suspending liquid of the unlaked suspension after addition of heated sera, 1.0123.

Counts with the Thoma-Zeiss haemocytometer gave for the unlaked suspension before addition of the sera 10,712,000 erythrocytes per cubic millimeter; and for the laked suspension, 7,196,000 ghosts. Allowing for the dilution by the added sera this gives 10,028,000 ghosts. Since it is practically impossible not to miss some ghosts the agreement is excellent, and it can be assumed that each erythrocyte is represented by a ghost.

In another experiment (February 20, 1925) with immune rabbit serum complete laking of a suspension of sheep's corpuscles containing 36.7 per cent of erythrocytes before addition of the serum (calculated from the conductivities) was accomplished

by addition to 25 cc. of the suspension of 4.5 cc. of the immune serum and 2.0 cc. of guinea pig's serum at 37°C. in 2 hours. The "reversal" phenomenon was well obtained. At the moment of addition of the serum the erythrocytes would constitute 29.1 per cent of the suspension. The volume of ghosts in the laked suspension, calculated from the conductivities, was 15.4 per cent. The values for $K\ (25°) \times 10^4$ were: unlaked suspension before addition of sera, 67.6; suspending liquid separated from this, 146.6; laked suspension, 75.9; liquid free from ghosts separated from laked suspension, 103.7.

The hemoglobin in the laked suspension was 71 per cent of Haldane's scale; in the liquid separated from it free from ghosts 70 per cent, and in the sediment 69 per cent. The difference in specific gravity between the laked suspension and the liquid separated from it was extremely slight.

A suspension with fewer sheep's erythrocytes was used in another experiment (February 19, 1925). The suspension was laked completely by addition of 3 cc. immune rabbit's serum and 1 cc. guinea pig's serum, kept at 40°C. for 1 hour. The reversal reaction was good. The erythrocytes constituted 16.8 per cent of the suspension and the ghosts 11 per cent of the laked suspension calculated from the conductivities, or allowing for the dilution by the sera 13.2 per cent (ratio 14:11). The hematocrit readings were 21 per cent for the erythrocytes and 18 per cent for the ghosts or allowing for the sera 21.6 per cent for the ghosts. No doubt the hematocrit readings are too large, especially for the ghosts. The hemoglobin in the laked suspension corresponded to 30 per cent of Haldane's scale; liquid freed fron ghosts 32 per cent; sediment 31 per cent. The specific gravities were: suspension (unlaked), 1.0228; liquid from unlaked suspension, 1,0063; laked suspension, 1.0230; liquid from laked suspension freed from ghosts, 1.0227.

In one experiment (March 2, 1925) a suspension of sheep's washed erythrocytes containing only one-tenth of the number of erythrocytes present in the original blood was laked by adding to 30 cc. of the suspension 0.1 cc. of immune rabbit's serum (1:10 dilution). After shaking and keeping it at 37°C. for 30 minutes, 1.5 cc. of guinea pig's serum was added. The suspension was again incubated for 45 minutes. Laking was complete, reversal good. The erythrocytes constituted 3.2 per cent of the unlaked suspension, calculated from the conductivities (4 per cent by hematocrit) and the ghosts 1.2 per cent of the laked suspension. The hemoglobin in the laked suspension was 8 or 7 per cent of Haldane's scale, in the liquid separated from the suspension, free from ghosts, 8 per cent and in the sediment 8 per cent.

Blood counts showed 1,099,000 erythrocytes in the unlaked suspension and 870,000 ghosts in the laked suspension per cubic millimeter, or allowing for the serum added, 916,000. A count of the ghosts in the laked suspension on the following day with colored light gave 983,000. The suspension was then put into the refrigerator and counts of the ghosts made each 24 hours for 5 days, with the following results: 1,006,000 (2 days); 606,000 (3 days); 627,000 (4 days); 473,000 (5 days); 446,000 (6 days from time of laking). It is clear that with this form of hemolysis, complete laking can be obtained with no loss of ghosts; also that the ghosts are only slowly destroyed or changed so as to become invisible.

Some experiments were made with dog's erythrocytes laked by the serum of rabbits immunised with dog's erythrocytes and guinea pig's serum. Satisfactory results, however, were not obtained because in each

case the immune serum in the quantity necessary for complete laking caused very strong agglutination, which of course interfered with the conductivity measurements. It was hoped that in time the agglutinating power would diminish sufficiently in proportion to the hemolysing power to enable the experiments to be carried out as planned. But although this did occur to some extent, agglutination was still too marked for our purpose. On the other hand no such difficulty presented itself in the laking of rabbit's corpuscles with dog's serum containing natural antibody. The only drawback was that a relatively large amount of the serum had to be employed. An example is given.

January 13, 1925. Rabbit's blood was washed twice with 0.85 per cent NaCl solution and the sediment made up to the original volume with the salt solution. 13 cc. of this suspension was laked by 9.75 cc. of dog's serum at 40° C. for 1 hour. Excellent pseudo-reversal obtained.

The values of K (25°C) $\times 10^4$ were: the unlaked suspension 67.5; the liquid separated from this suspension 149.3; the laked suspension 74.9; the liquid separated from the laked suspension freed from ghosts 120.3; the unlaked suspension *plus* the same proportion of dog's serum heated to 60°C. as was added of unheated serum in the laking observation (no laking) 87.1; the suspending liquid separated from the unlaked suspension *plus* 0.75 cc. of dog's serum added to each cubic centimeter 141.5; the dog's serum, 125.9; the dog's heated serum, 125.3.

It was calculated from the conductivities that the unlaked suspension of rabbit's corpuscles in salt solution contained 37.7 per cent of erythrocytes. The laked suspension contained 22.3 per cent of ghosts. The percentage of erythrocytes in the original (unlaked) suspension, allowing for its dilution to the same extent as the laked suspension by the dog's serum, would be 21.5 per cent. The volume of ghosts is thus practically the same as that of the erythrocytes.

The hemoglobin in the laked suspension was 44 per cent of Haldane's scale; in the liquid separated from the laked suspension and free from ghosts 43 per cent; in the sediment from the laked suspension 44 per cent. Allowing for dilution with the dog's serum this would correspond to 77 per cent of the scale for the hemoglobin in the unlaked suspension, or 204 per cent for the erythrocytes of that suspension. If we suppose that 100 cc. of erythrocytes contained 26 grams (say, 20 cc.) of hemoglobin before laking this would be reduced after laking to 5.6 grams (say, 4.3 cc.) in the ghosts. In the laking process the stromata must therefore have taken up on balance water amounting to about 16 per cent of the original volume of the erythrocytes. The relatively small proportion of hemoglobin in the ghosts is of course due to the great dilution of the suspension by the dog's serum.

The number of erythrocytes and ghosts compared with different methods of laking. The observations described show that with the methods of laking studied (oleate, heat, saponin and specific antibody) large numbers of ghosts are present after complete laking, and that the total volume of the ghosts may even be quite equal to that of the unlaked erythrocytes. This has been emphasized by one of us repeatedly because so many writers, who apparently do not know how to recognize ghosts or have failed to look for them, have assumed that the erythrocytes are as a matter

of course "dissolved." With the forms of hemolysis mentioned as well as laking by water, by freezing and thawing and in other ways, it can be seen that after complete laking the field is crowded with ghosts. But the only way to know whether any have disappeared is to count them. Even with good technique and much practice it is probable that some ghosts are bound to be omitted in the count. It is therefore to be expected that a certain deficit should be found. It will be seen from our results that with some of the forms of laking, (especially specific antibody, water, higher fatty acid, or soap laking) the deficit is of such an order as to warrant the conclusion that practically none of the stromata are completely dissolved, or altered so much as to be invisible. With saponin the deficit was more considerable, but it is possible that if the dose just necessary for complete laking were hit more exactly, the deficit might be reduced. The results obtained with linolenic acid did not differ from those seen with sodium oleate. Rabbit's blood seemed to require somewhat more linolenic acid for complete laking than Brinkman used as a minimal dose for pig's blood. Good pseudo-reversal was got with rabbit's blood with all the different reagents used by us in other forms of laking.

Some of the counts with the Thoma-Zeiss hemacytometer are given in the table. "Rabbit's erythrocytes" or "sheep's erythrocytes" in the first column means that erythrocytes twice washed with 0.85 per cent NaCl solution were used. The sediment of erythrocytes was generally made up with the salt solution to 10 times the original volume of the blood, occasionally to twice the original volume or to the original volume. The erythrocyte and ghost count are corrected for the dilution, i.e., expressed as the numbers which would have been present had the suspensions always been made up to the original volume of the blood. The numbers given are generally averages of two, three or more separate counts. In the first two experiments in the table the same suspension of rabbit's erythrocytes was used for heat and for water laking. Where defibrinated blood was used it was undiluted in the examples included in the table. When any dilution was caused by the laking agent it was taken account of.

As regards the technique it is unnecessary to say much. Some practice is essential. Yellow light makes the ghosts more visible and facilitates counting. As uniform a distribution of the ghosts as possible is of course important. Clumping renders the count abortive. The nature of the diluting fluid is of considerable influence. If it is too strongly hypertonic the ghosts are liable to clump. If it is decidedly hypotonic there is a chance for further (water) laking. Distilled water was used for a time but was abandoned for this reason. Also it did not increase the visibility of the ghosts. A large number of solutions were tried. A mixture of 7 cc. distilled water, 2 cc. of 4 per cent formalin, and 1 cc. of $\frac{M}{2}$ Na_2SO was found to be good.

MATERIAL	METHOD OF LAKING	ERYTHRO-CYTES	GHOSTS	GHOSTS NEXT DAY
Rabbit's erythrocytes 10%	Heat	5,992,000	4,520,000	4,760,000
Rabbit's erythrocytes 10%	Water	5,992,000	5,230,000	4,500,000
Rabbit's erythrocytes 10%	Na oleate	4,444,000	3,062,000	
Rabbit's erythrocytes 10%	Saponin	5,108,000	3,224,000	3,668,000
Rabbit's defib. blood	Linolenic acid	6,650,000	5,860,000	
Rabbit's defib. blood	Mechanical	6,660,000	132,000*	
Sheep's erythrocytes 10%	Rabbit's immune serum	10,992,000	9,340,000	9,564,000
Sheep's erythrocytes 10%	Rabbit's immune serum	10,990,000	9,160,000	9,830,000†
Sheep's erythrocyres 100%	Rabbit's immune serum	10,712,000	10,028,000	
Sheep's erythrocytes 50%	Dog's serum	8,248,000	8,824,000	
Sheep's erythrocytes 100%	Heat	10,836,000	7,688,000	

* In addition about the same number of unlaked erythrocytes were present.

† One week later, the material having been kept in refrigerator, the ghost count was 4,244,000.

Both the Thoma-Zeiss chamber (0.1 mm. deep) and the Helber cell (0.02 mm. deep) were used. It was thought that the latter, giving practically a single layer of ghosts, would be the more accurate. But although it was faithfully tried, and a much larger number of squares were counted to make up for the smaller number of ghosts in a field, it was found that for one reason or another the results were no better than with the Thoma-Zeiss cell, perhaps not as good.

Defibrinated blood when laked is viscous, and the ghosts have a tendency to clump, which makes accurate counting impossible. Accordingly we generally used suspensions of washed corpuscles made up to from 3 to to 10 times the original volume of the blood. Even then heat-laked corpuscles are sticky, tend to clump and are difficult to count.

With so-called mechanical laking it depends upon the method employed what proportion, if any, of the stromata will survive the laking process. In two experiments on rabbit's corpuscles we tried rubbing up with sand. Complete laking was produced, but so many of the stromata were destroyed that good pseudo-reversal was not obtained. Further, the sand interfered with the conductivity measurements, and we did not succeed in separating it completely without losing a portion of the sediment of ghosts and fragments of ghosts.

With de Waard's (1923) apparatus no completely satisfactory results for our purpose were obtained. That does not imply any depreciation of this ingenious method for other purposes. What we aimed at, and what was accomplished with oleate, water, heat, immune serum and other forms of hemolysis was complete laking of the erythrocytes without destruction of the stromata. Where the corpuscles are forced under pressure through

a space many times too small for them many of them are necessarily ruptured and destroyed. If any intact erythrocytes come through, this is simply because the contact of the steel ball and the tube is not equally close at all points. We improved the apparatus in certain ways, although still it did not serve our purpose. A side opening was made in the tube, through which it was refilled without completely withdrawing the plunger. In the original form it was found troublesome to pull the plunger out. This difficulty was obviated by making the plunger screw in, instead of being pushed in. The packing was attached to the tube instead of being attached to the plunger, heavy cord being used instead of leather. In this way leakage was prevented.

One experiment was made with dog's blood. But when it came out it was nothing but a mass of hemoglobin crystals and could not be used.

Rabbit's blood was free from this complication. But the total volume of ghosts was not large and many were broken into fragments. These experiments may be illustrated by the following protocol.

February 13, 1925. Rabbit's defibrinated blood was passed through the apparatus. Laking was practically complete. Fair pseudo-reversal. The values of K (25°) $\times 10^4$ were: defibrinated blood, 69.3; serum, 128.9; the laked blood, 91.7; liquid from the laked blood freed from sediment, 102.9. The erythrocytes made up 29.3 per cent of the volume of the unlaked blood, as calculated from the conductivities; the laked blood contained only 9.6 per cent of suspended material. The hematocrit gave 10 per cent of sediment in the laked blood. The hemoglobin in the laked blood was 88 per cent of Haldane's scale; in the liquid from the laked blood freed from suspended matter 88 per cent; in the sediment 86 per cent. The hemoglobin in the unlaked erythrocytes can be calculated to have been about 35 per cent of the moist weight. The specific gravity of the defibrinated blood (unlaked) was 1.0526; of the laked blood, 1.0529; of the serum, 1.0273; of the liquid from the laked blood freed from suspended material, 1.0501.

In another experiment rabbit's blood was diluted with an equal volume of 0.85 per cent salt solution. It was practically completely laked when forced through the apparatus, but pseudo-reversal was only fair and many ghosts had been disrupted. The values for K (25°) $\times 10^4$ were: the unlaked diluted blood, 89.4; the liquid separated from this, 144.9; the laked diluted blood, 106.0; the liquid separated from this freed from suspended material, 120.0. The erythrocytes made up 22.7 per cent of the volume of the unlaked suspension. The suspended material in the laked blood constituted 10.3 per cent of the total volume. The corresponding hematocrit readings were 29 per cent (not carried to the end point) and 11 per cent. The hemoglobin in the laked blood was 54 per cent of Haldane's scale, in the liquid from the laked blood free from suspended material 54 per cent. The hemoglobin must have constituted about 28 per cent of the weight of the moist erythrocytes.

Oligo-dynamic action of metals. A few observations were made on the effect of bright copper wire and of steel wool on blood, serum, and suspensions of washed rabbit's erythrocytes. The so-called oligo-dynamic actions of metals on blood have been studied by a number of observers, e.g., Saxl (1917), and Hess and Reitler (1921). We were especially

interested in the microscopic study of the ghosts and pseudo-reversal, and in the conductivity changes, which have not hitherto been investigated. Erythrocytes suspended in isotonic salt solution are laked when a coil of thin, bright copper wire is left in the suspension for a time. Serum is said to inhibit the action and this inhibition may be connected with the precipitation of proteins caused by copper wire in serum. However, serum does not entirely protect, and laking of blood can also be produced by copper wire. The blood or suspension is darkened, and if the action is allowed to go on the blood pigment becomes brownish, and is precipitated. Under certain conditions it appears that precipitation of some of the pigment occurs within the erythrocytes. The ghosts are characterized under the microscope by an exceedingly sharp dark contour, which renders them very distinct. Typical reversal was not observed, although the ghosts shrink upon the addition of the solutions which cause reversal with other forms of laking. Possibly the blood pigment is so changed that the ghosts cannot assume a normal appearance. We can confirm the statement that erythrocytes are laked in isotonic salt solution if the salt solution has been in contact with copper wire for some time. Conductivity measurements showed that only an exceedingly small quantity of copper could have gone into solution. But the very delicate diphenylcarbazide test said to be capable of detecting copper in the dilution of 1 part in 2 million parts of water, gave a positive result with water and with 0.85 per cent NaCl solution, in which copper wire was left overnight at room temperature. Serum was darkened and coagulated. Copper wire left in blood overnight caused laking, coagulation of hemoglobin and darkening.

Rabbit's defibrinated blood, diluted with an equal volume of 0.85 per cent NaCl solution, was left in contact with copper wire for 20 hours at room temperature. The value for K (25°) $\times$ 10^4 was 96.8. For the same blood mixture kept under similar conditions without copper wire the conductivity was 79.0. The conductivity of the 0.85 per cent NaCl solution in contact with copper wire for 20 hours was 150.2; of the suspending liquid separated from the diluted blood not in contact with copper 144.9. When this suspending liquid was left for 20 hours in contact with copper wire its conductivity was 145.7. It is evident that the increase in conductivity of the laked blood mixture could not have been due to solution of copper but must have been due to the laking.

A similar action on blood was produced by steel but the color was blacker.

SUMMARY

It was shown by one of us (partly in collaboration with Peskind) in 1901 and 1902 that apparent reversal of hemolysis may be produced in erythrocytes (Necturus and mammalian) under conditions which preclude the passage of blood pigment into the corpuscles from the outside. Ery-

throcytes fixed by formaldehyde when heated in ammoniacal water become pale, as if laked, but when now acted upon by various solutions *appear* to re-accumulate the pigment. It is easy to show that the pigment has never been outside them. These observations have been extended in the present research to the large erythrocytes of Cryptobranchus, with the same result.

By microscopic observations it is demonstrated in various ways that unfixed erythrocytes when laked may be caused to become colored again when it is not possible that all, or in certain cases any, of the pigment can have re-entered the ghosts from the outside. At the same time objects which have never contained hemoglobin may seem sometimes to take it up and to appear pigmented when acted upon by certain solutions (nuclei of nucleated erythrocytes, cytoplasm of leucocytes and of epithelial cells). Here there is reason to believe that the phenomenon is an artificial one, without biological significance, and depending upon the previous imbibition of the cells with the hemoglobin solution and the retention and concentration of hemoglobin already within them at the moment when they are acted upon by the solutions which cause apparent "reversion."

The main portion of the research consisted of quantitative experiments on rabbit's and sheep's blood (either the defibrinated blood or suspensions of washed erythrocytes, or both). In these were determined: *a*, the electrical conductivity of the blood or suspension, of the serum or suspending liquid and sometimes of the sediment, before and after laking by sodium oleate, heat, saponin or immune serum; *b*, the hemoglobin concentration in the same fractions; *c*, the specific gravity of the same fractions.

From the conductivities was calculated the minimum proportion of the total volume of the laked suspensions (or blood) occupied by the ghosts. The calculated volumes were checked by hematocrit readings although for obvious reasons the degree of accuracy attainable with the hematocrit for ghosts is still less than for erythrocytes. The hemoglobin concentrations were practically the same for the laked suspensions, the suspending liquids separated from them and freed from ghosts, and the sediment of ghosts. The specific gravities were not much different for the suspending liquid of the laked material freed from ghosts, the laked suspension and the sediment of ghosts. But as follows from the fact that sedimentation was obtained by centrifuging, they increased in the order named.

From the substantial proportion of the total volume occupied by the ghosts, according to *a*, (with gentle laking agents sometimes not less than that occupied by the unlaked erythrocytes) and the findings under *b* and *c*, it follows that the volume of the ghosts in the laked suspension must be made up mainly of approximately the same hemoglobin solution as that in which they are suspended. After complete laking, therefore, the ghosts contain a relatively large amount of hemoglobin, and the apparent

"reversal" of hemolysis, when they are acted upon by solutions of various substances, is essentially due to an alteration in the condition (concentration) of hemoglobin already within them and not to the taking up of hemoglobin from the outside at the moment of "reversal."

It is shown how information can be derived from the conductivities and hemoglobin concentrations of the various fractions as to the exchange of water and electrolytes between the corpuscles and the suspending liquid in hemolysis.

Counts of the erythrocytes and ghosts in various forms of hemolysis were made in order to determine quantitatively whether any of the ghosts were completely destroyed. While there was usually a deficit in the number of ghosts in laking by immune serum, water, linolenic acid, sodium oleate and heat, this is partly at least only an apparent deficit due to the difficulty, or where there is clumping, as in heat laking, the impossibility of counting all the ghosts. It is certain that in the forms of laking mentioned and also in saponin hemolysis vast numbers of ghosts remain after complete laking. Only in one form investigated by us (mechanical laking) was there unequivocal evidence of stromata being destroyed in large numbers.

A few observations were made on the so-called oligo-dynamic action of metals on blood. The ghosts after the action of copper wire were extremely well defined.

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CHANGES IN THE PHYSIOLOGICAL ACTION OF INSULIN INDUCED BY EXPOSURES TO ULTRAVIOLET LIGHT

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Commercial insulin (Lilly Iletin, U-20 and U-40) was used in all of these experiments, either undiluted or reduced to 10 clinical units per cubic centimeter by the addition of sterile physiological saline which had been boiled previously to drive off all air in solution. In each test the insulin solution was divided into three equal parts two of which were placed at once into pyrex test tubes and the third into a transparent, fused-quartz test tube. All test tubes were approximately 1 mm. in thickness and 20 mm. in diameter. These test tubes were quickly sealed with cork stoppers which had been boiled in paraffine, and the air driven out of each tube by nitrogen gas which was allowed to flow in and out for several minutes through two small glass tubes sealed into the cork stopper. These small glass tubes were finally sealed off and the insulin solution left in an atmosphere of pure nitrogen. This substitution of nitrogen for air was made to eliminate the free oxygen and the ozone which might be formed from it in the presence of ultraviolet light, as Shonle and Waldo (1923) have pointed out that insulin is easily destroyed by the oxidative action of hydrogen peroxid and potassium permanganate. As the saline with which the insulin solutions were diluted was air-free, and as the nitrogen streamed over the insulin solution for several minutes before the tubes were finally sealed, the only free oxygen present in the tubes at the time they were exposed to the ultraviolet light was a very minute quantity which might have been held in solution in the original undiluted iletin and which was not replaced by nitrogen during the time that gas streamed through the tubes. After the test tubes were filled with nitrogen and finally sealed, one pyrex tube was put aside in a dark closet at room temperature (20 to 30°C.) as a control, and the other two tubes, i.e., one pyrex and one quartz, were placed in the ultraviolet light.

The ultraviolet light was produced by a Cooper-Hewitt mercury vapor lamp, operating at 7.5 amperes and 125 to 130 volts. All tubes were exposed at a distance of four inches from the lamp and were constantly cooled by an air current from an electric fan. The thermometer readings

showed that the temperature of the fluid in the test tubes never exceeded 35°C., the average temperature being about 30°C. throughout the period of exposure.

Changes in the insulin solutions. There were no apparent changes in the physical properties of the insulin solutions held under nitrogen as controls, and the physiological action of these controls proved that there had been no change in potency of the insulin contained.

The solutions exposed to the ultraviolet light in quartz tubes did however develop definite color after four hours under the lamp. The fluid became slightly opalescent and exhibited a faint rosy florescence when shaken in ordinary daylight. This rosy florescence was even more evident in the greenish-blue light of the mercury vapor lamp. The solution as a whole had a distinctly brownish cast. After 24 hours' exposure to the ultraviolet light the insulin solution was still slightly opalescent, but not turbid, as print could be read through this brownish solution in spite of its color, quite as easily as through an equal volume of unexposed insulin solution. The exposed solution by the end of the 24th hour had developed a pale testaceous brown color with evident red and black qualities, the reddish florescence being about the same as that noted at the end of the 4th hour of exposure. Forty-eight hours' continuous exposure made little additional change in the color of the solution, but some time after the 44th hour a very fine precipitate began to settle out. This precipitate was present only in very minute quantities and was so finely divided that after slight agitation of the test tube the precipitate remained in suspension for hours.

The insulin solutions in pyrex tubes exposed to the ultraviolet light showed no change at the end of the fourth hour and very little if any at the end of 24 hours. By the end of the 48th hour of exposure the solutions had developed traces of the brownish color and of the reddish florescence noted in the solutions exposed in quartz. No precipitate was seen in any pyrex tube regardless of the length of exposure up to 48 hours, and it was evident that the changes induced by the ultraviolet light were proceeding at a much slower rate in the pyrex tubes than in the quartz tubes. This of course was to be expected as the pyrex glass screened out practically all of the shorter rays.

As the pH value of certain solutions is known to change in the presence of ultraviolet light, the pH values of the unexposed control which had been held under nitrogen, and of the solutions from both pyrex and quartz tubes after 24 hours' exposure to the light, were determined electrometrically in a series in which Lilly Iletin U-40 had been diluted to 10 clinical units per cubic centimeter. The pH value of the unexposed control was 3.6, that of the exposed solution from pyrex 3.4, and that from the quartz tube 3.1. As all of these tubes contained aliquot parts of the same original

dilution the differences in pH value, although slight, suggest an increase in acidity in the insulin solution following exposure to ultraviolet light.

Physiological tests. The action of the exposed and unexposed insulin solutions as measured by changes in the blood sugar level, was tested on dogs, cat, and rats, all animals being in the post-absorptive stage, i.e., 18

TABLE 1

Adult cats

WEIGHT	CLINICAL UNITS PER KILOGRAM	NORMAL BLOOD SUGAR	BLOOD SUGAR FOLLOWING INJECTION			
			1½ hours	4 hours	6 hours	24 hours
I. Unexposed insulin controls						
kgm.						
2.1	10	0.092	0.070	0.066*	0.058*	0.110
2.4	10	0.090	0.062*	0.054*	0.058*	0.090
II. Exposed insulin in pyrex						
24 hours' exposure						
2.0	10	0.094	0.058*	0.056*	0.052*	0.102
3.0	7	0.108	0.042*	0.046*	0.070	0.100
48 hours' exposure						
3.0	10	0.108	0.088	0.060*	0.078	0.106
2.7	10	0.105	0.078	0.060*	0.070	0.110
III. Exposed insulin in quartz						
4 hours' exposure						
3.5	10	0.110	0.068	0.056*	0.080	0.106
2.5	7	0.108	0.065	0.048*	0.084	0.098
24 hours' exposure						
3.0	10	0.096	0.100	0.102	0.126	0.100
3.0	10	0.100	0.120	0.096	0.090	0.098
48 hours' exposure						
3.0	10	0.106	0.116	0.142	0.124	0.110
2.0	10	0.101	0.104	0.140	0.110	0.105

* Animal prostrate and in convulsions.

hours since last feeding, when injected. Blood samples were taken from the ears of the dogs and cats, the blood being allowed to drop freely into a paraffine cup. In the rat series where only the final blood sample was taken, each animal was lifted quickly from its cage and stunned by a sharp blow on the head. The animal was decapitated immediately after being

struck and the blood sample collected in paraffine from the neck arteries. Typical series of each sort are given in tables 1, 2, 3 and 4.

TABLE 2

Adult dogs

WEIGHT	CLINICAL UNITS PER KILOGRAM	NORMAL BLOOD SUGAR	BLOOD SUGAR FOLLOWING INJECTION		
			1½ hours	4 hours	24 hours
I. Unexposed insulin controls					
kgm.					
10.5	6.5	0.108	0.100	0.060	0.112
10.0	6.5	0.100	0.070	0.050	0.115
16.0	5.0	0.100	0.080	0.069	0.080
II. Exposed insulin in quartz					
4 hours' exposure					
14.0	6.0	0.100	0.131	0.131	0.110
9.0	6.0	0.100	0.101	0.120	0.090
24 hours' exposure					
17.0	6.0	0.110	0.120	0.148	0.105
10.5	6.0	0.111	0.115	0.165	0.120
8.0	5.0	0.080	0.100	0.117	0.090
16.0	5.0	0.106	0.110	0.114	0.090

TABLE 3

Adult rats

Blood sugars 4 hours after injection

PHYSIOLOGICAL SALINE CONTROLS	UNEXPOSED INSULIN CONTROLS	EXPOSED INSULIN	
		44 hours in pyrex	24 hours in quartz
0.102	0.070	0.060	0.105
0.105	0.075	0.064	0.120
0.110	0.080	0.066	0.124
0.110	0.085	0.067	0.144
0.114	0.090	0.085	0.150
0.116	0.090	0.088	0.156
0.116			
Av. 0.110	0.081	0.071	0.133

Each animal was injected with ¼ cc. of fluid and each ¼ cc. of the various insulin solutions carried 2.5 clinical units.

From the data in these tables it is apparent that the unexposed insulin controls did not lose potency when kept under nitrogen for 48 hours or less, as animals injected with usual doses of insulin from the control tubes

developed hypoglycemia and convulsions at the same time and to the same degree as would be expected from similar injections of commercial insulin.

The insulin exposed in quartz to the action of the ultraviolet light for 19 hours or longer, and in one series that exposed for only four hours, did

TABLE 4

Adult cats

WEIGHT	CLINICAL UNITS PER KILOGRAM	NORMAL BLOOD SUGAR	BLOOD SUGAR FOLLOWING INJECTION			
			1½ hours	4 hours	6 hours	24 hours
I. Reprecipitated insulin, unexposed, sealed in nitrogen for 19 hours						
kgm.						
3.0	10	0.098	0.056*	0.058*	0.070	0.100
2.5	10	0.105	0.058*	0.056*	0.066*	0.108
II Reprecipitated insulin, unexposed, treated with oxygen and ozone for one hour, sealed in oxygen for 18 hours						
3.0	10	0.110	0.094	0.106	0.110	0.112
2.5	10	0.108	0.100	0.112	0.104	0.100
III. Reprecipitated insulin, exposed in quartz 19 hours, sealed in nitrogen						
3.0	10	0.100	0.124	0.120	0.120	0.110
3.0	10	0.094	0.136	0.138	0.120	0.108

* Prostrate and in convulsions.

TABLE 5

Adult rats

Blood sugars 4 hours after injection reprecipitated insulin

UNEXPOSED INSULIN CONTROLS	OZONE-TREATED INSULIN	INSULIN EXPOSED FOR 19 HOURS IN QUARTZ
0.060	0.110	0.160
0.060	0.110	0.156
0.058	0.104	0.154
0.055	0.100	0.124
0.052	0.094	0.120
0.050	0.090	0.100
Av. 0.056	0.101	0.134

Each animal was injected with ¼ cc. of fluid carrying approximately 2.5 clinical units.

not lower blood sugar nor produce convulsions but on the contrary raised the blood level, i.e., produced a hyperglycemia. This action was universal in all lots run if the exposure exceeded four hours. The animals

injected with this exposed insulin showed no signs of convulsions or other distress, seemed in general good condition, but were possibly a little more inclined to sleep than uninjected animals kept under the same conditions. The difference between the physiological action of the insulin exposed for four hours (table 1) which retained its power to produce hypoglycemia and that (table 2) which lost its hypoglycemic power in a four-hour exposure, is probably a function of the quartz tubes in which these samples were exposed. It is well known that different quartz tubes have different degrees of transparency to the ultraviolet light, and the tubes used in experiments 1 and 2 were made by different manufacturers. This difference in the quartz tubes themselves which would alter the amount of active rays reaching the insulin solution together with the fact that different lots of insulin were used in experiments 1 and 2 would account for the difference in actual time required to produce the changes in the insulin solution, but the end results are the same regardless of the tube used if the exposure were long enough, the insulin losing its power to produce hypoglycemia, the injection of the exposed solution inducing on the contrary a hyperglycemia.

The insulin solutions exposed in pyrex tubes remained potent producing marked hypoglycemia on injection after 48 hours under the mercury lamp, but on a quantitative basis the 48-hour samples seemed slightly less potent than unexposed control insulin.

As commercial Lilly Iletin contains a fraction of a per cent of tricresol as a preservative, reprecipitated insulin tricresol-free was also exposed to the action of the ultraviolet light to determine whether the tricresol was a factor in these changes. Reprecipitated insulin was prepared by precipitating commercial insulin (Lilly U-20) isoelectrically, using dilute sodium hydroxid to change the pH value of the solution. The iso-electric precipitate was collected after flocculation by centrifuging. This precipitate after the removal of the supernatant liquid was taken up in distilled water which had been rendered slightly acidic by the addition of a small amount of dilute hydrochloric acid, and a second isoelectric precipitate again thrown down by the addition of dilute sodium hydroxid. This second precipitate was collected after flocculation as before and was redissolved in sterile physiological saline adjusted to a pH of about 3.5 with dilute hydrochloric acid. To eliminate the action of any free oxygen as far as possible the saline was boiled to remove any dissolved air and as soon as the almost dry precipitate was taken up in this saline the sealed tubes were flooded with nitrogen gas, which streamed through for several minutes. As an additional check the nitrogen gas was driven through a strong solution of pyrogallic acid before entering the tubes containing the insulin solution, to remove any traces of oxygen which might be mixed with the nitrogen. The reprecipitated insulin was divided into

three equal parts as before, two in pyrex and one in quartz. One of the pyrex tubes was put aside under nitrogen as a control, and the other pyrex tube instead of being exposed to the ultraviolet light was used as an additional check on the action of free oxygen and ozone. This pyrex tube was connected with an ozonator and a mixture of free oxygen and ozone bubbled through the insulin solution for one hour, the bubbles breaking at the rate of three per second. This tube was then sealed with the contained oxygen and ozone and put aside in a dark closet with the nitrogen control for 18 hours. The quartz tube meanwhile was exposed to the ultraviolet light for 19 hours, after which all tubes were tested.

The blood sugar changes of six adult cats injected with insulin solutions from the reprecipitated series are given in table 4. The reprecipitation of insulin for this and other experiments has been carried out by us repeatedly following the method given above, without loss of potency, and aside from the actual loss of some of the material during the process of transfer and centrifuging the insulin has been recovered very largely. Consequently no change in the potency of the unexposed but reprecipitated insulin was expected. The readings in group I of table 4 show that this reprecipitated insulin did produce typical hypoglycemia when injected and the test animals displayed the characteristic convulsions.

One hour treatment with a mixture of free oxygen and ozone followed by 18 hours under oxygen and ozone practically if not completely destroyed the power of the insulin so treated to produced hypoglycemia. Cats injected with this substance did show a slight fall in the blood sugar level during the first hour and a half after injection, but the change was so small that little significance was attached to it. It is possible that all of the insulin was not destroyed by the oxygen-ozone treatment and that a small amount of uninjured insulin was responsible for this slight fall, if it be considered a valid fall, in the blood sugar level. The oxygen-ozone test is introduced in this discussion however only as it has bearing on the ultraviolet light reactions. The absence of any hyperglycemic reaction following the injection of the ozone-treated insulin was conspicuous in both cat and rat series, and is in contrast to the hyperglycemia produced by the insulin exposed in quartz tubes, both in the experiments with reprecipitated insulin and those in which diluted iletin was used. From the tests made with the reprecipitated insulin it seems evident that the ultraviolet light is at least one of the active factors in changing the insulin exposed in quartz tubes from a solution which produced hypoglycemia to one which produce hyperglycemia. The presence of tricresol is not essential to this change and ozone in ordinary daylight, that is, without the help of the light from the mercury lamp, merely destroyed the power of the insulin to produce hypoglycemia without developing a hyperglycemia-producing solution. It is recognized that minute quantities of free oxygen may have

been present in the quartz tubes containing the insulin solutions when these were exposed to the ultraviolet light although these quantities of oxygen must have been extremely small, and the apparent action of the ultraviolet light may have been therefore the combined action of the light and these traces of oxygen. It is also possible that the ozone-treated insulin failed to give any hyperglycaemia as a result of overoxidization by the ozone since much larger quantities of ozone were used in the preparation of the ozone-treated insulin than could have been produced from the traces of oxygen which might have been left in the quartz tubes exposed to the ultraviolet light. However, whether the changes in the insulin solutions exposed in quartz to the ultraviolet light were the result of minute oxidations induced by the action of the ultraviolet rays on traces of oxygen which may have been present in these tubes, or whether the changes followed some more specific action of the ultraviolet light alone, all of the tests agree in showing that the power of insulin to produce hypoglycemia is destroyed by sufficient exposure to ultraviolet light in quartz tubes, and that some substance or substances are developed in the solution which produce a hyperglycemia when injected into test animals.

The action of ultraviolet light on insulin solutions is therefore suggestive in connection with the hyperglycemia-producing and toxic fractions which Fisher (1923) and others have separated from relatively well purified insulin preparations, for whether the action of ultraviolet light on insulin be direct or indirect through induced oxidative changes, these possibilities are not altogether excluded in the preparation of insulin at the present time.

While this work was in progress a short article by Nitzescu (1924) appeared in which it is stated that insulin exposed in vacuo in "Uviolglas" containers to ultraviolet light for from one to three hours was not changed. These figures agree with our own as the shortest exposure which changed the insulin under the conditions of our experiments was four hours in quartz and as pointed out above, even four-hour exposures were not sufficient to bring about these changes in insulin exposed in one of our tubes owing to a difference in the transparency of that tube to the ultraviolet light. It seems therefore that Nitzescu's experiments were not carried far enough. He gives but a single case, that of a rabbit which was injected with insulin which had been exposed for three hours, which test is interesting in another connection. This animal showed a fall of blood sugar from 0.101 to 0.044 in two and one-half hours following the injection of one "Toronto Unit" of the exposed insulin, although the rabbit weighed almost two kilos. This fall in blood sugar indicates that the exposed insulin was not only potent after the exposure, but very potent in terms of ordinary commercial insulin. In our tables 1 and 3 a slight quantitative difference in the potency of the exposed insulin as compared with the unexposed control insulin, in favor of the exposed insulin, may be

seen in the shorter and less complete exposures, that is, insulin exposed for four hours in the less transparent quartz tube, and insulin exposed in pyrex glass which filters out almost all of the short rays, for 24 to 44 hours, seemed slightly more potent than the unexposed control insulin. Other experiments not yet published suggest this same variation not only for insulin solutions but for glucokinin solutions prepared from rice, following non-destructive exposures to ultraviolet light, so that there may be an "activating" action of ultraviolet light preceding the changes which destroy the power of the insulin to produce hypoglycemia and which develop the hyperglycemia-producing solution. Owing to the factor of individual variation of the test animals this is offered only as a suggestion until confirmed or disproved by large series of tests.

SUMMARY

1. Insulin solutions exposed in an atmosphere of nitrogen in transparent fused-quartz test tubes to the action of the ultraviolet light from a mercury vapor lamp for periods of from 19 to 48 hours lost their power to produce hypoglycemia when injected into test animals.

2. Some substance or substances were developed in these exposed insulin solutions which produced hyperglycemia when injected.

3. Insulin solutions under nitrogen in pyrex glass test tubes which filter out most of the short rays did not lose their power to produce hypoglycemia when injected into test animals, after 48 hours' exposure under the mercury vapor lamp, although quantitatively the 48-hour samples seemed slightly less potent than the unexposed controls.

4. These changes in the insulin solutions were not due to the presence of tricresol.

5. Ozone and free oxygen in ordinary daylight destroyed the power of unexposed insulin solutions to produce hypoglycemia, but did not develop hyperglycemia-producing substances in one-hour treatments.

6. A possible "activating" action of ultraviolet light on insulin in non-destructive exposures is mentioned.

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ABSORPTION OF UNDIGESTED PROTEIN FROM THE ALIMENTARY TRACT AS DETERMINED BY THE DIRECT ANAPHYLAXIS TEST

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It is generally held that normally all proteins are absorbed into the blood in the form of amino acids. Nevertheless it is conceded that whole undigested protein may, under certain conditions, find its way into the circulation (Fischer, 1921), (Hammersten, 1909), (Mathews, 1921).

The demonstration of the absorption and presence in the blood of undigested protein rests upon the specificity and delicacy of the precipitin, the fixation of complement and the anaphylaxis tests as applied to the blood and urine (Ascoli, 1902), (Croftan, 1908), (Wells, 1909), (Van Alstyne and Grant, 1911). Recently the coagulation-time test has been used by Mills, Dorst, Mynchenberg and Nakayama (1923) in connection with the absorption of tissue fibrinogen.

Results based on these tests have been somewhat conflicting. For example, the anaphylaxis test for egg albumen as applied to the urine after ingestion of large amounts gave negative results in the hands of Wells (1909), whereas Van Alstyne and Grant (1911) obtained positive results in both blood and urine after injecting egg albumen directly into Thiry-Vella fistulae of the intestine. Apart from the difference in the administration of the protein in these two investigations, it is to be noted that the procedure of testing for the suspected protein in the blood or urine was the same. A small amount of blood or urine from the patient or experimental animal was injected for sensitization into a guinea pig. After two weeks or more, an intoxicating dose of the suspected protein (egg white) was injected intraperitoneally into the test animal resulting in either positive or negative symptoms of anaphylaxis.

It seemed to us that the positive or negative result of the anaphylactic reaction would be equally spectacular and perhaps more convincing if the experimental animal were at the same time the test animal; that is to say, if the absorption and presence in the blood of a certain undigested protein were at once made manifest by anaphylactic symptoms, the animal having been previously sensitized to the particular protein. This might be called

the direct anaphylaxis test in contradistinction to the indirect method as used by others.

It was realized that the test in the above form would not be as delicate as when the blood of the experimental animal is injected into a test animal for sensitization because the dose required for sensitization is far less than that required for intoxication. In our form of the test an amount of undigested protein sufficient for intoxication must be absorbed. Although we performed experiments demonstrating sensitization by the intestinal route, our primary interest lay in finding the necessary conditions for intoxication by that route. The considerations just stated provided the basis for the experiments here reported.

EXPERIMENTAL. The guinea pig was chosen for the experimental and test animal chiefly because the overt symptoms of anaphylaxis are pronounced and easily recognized in that animal. The usual progression of symptoms is hyperactivity, rapid chewing movements, pawing at the mouth, urination and defecation, paralysis of the hindquarters, more or less complete paralysis and death. The degree of intoxication is indicated by the severity of the symptoms and found to be dependent on the foreignness of the protein, the quantitative relationship between sensitizing and intoxicating dose, the interval elapsing between the administration of each, the route of administration, and the age of the animal. Most of these factors were controlled by always employing animals of nearly the same age (two months), the same sensitizing dose (0.01 cc. horse serum) via the same route (intraperitoneal) and practically the same intoxicating dose (1.0 to 2.0 cc. horse serum) after a definite number of days (twenty-one). For the intraperitoneal administration of the toxic dose in control experiments the symptoms regularly began in 30 to 35 minutes after the injection. The severity of the symptoms was remarkably constant. There were always rapid chewing movements, hyperactivity, pawing at the mouth, urination and defecation and some paralysis of the hind quarters, but rarely complete paralysis and death.

A. *Intestinal injection of horse serum in small amounts.* A guinea pig sensitized three weeks previously with 0.01 cc. horse serum in 0.99 cc. physiological salt solution, was anesthetized with ether, a 2 cm. incision made through the abdomen and about a 10 cm. loop of small intestine drawn forth. Into this loop 2.0 cc. of undiluted horse serum were injected, the loop replaced, the wound sutured and the animal allowed to come out of the anesthetic. After this the animal was kept under observation for one to two hours, particular attention being directed to the appearance of signs of anaphylactic shock. It is to be noted that guinea pigs frequently make vigorous scratching movements with the hind feet in coming out of the ether. Such movements are not to be mistaken for a symptom of anaphylactic shock. The pawing or scratching in the latter case in-

volves primarily the front feet, rarely the hind feet and begins moreover after a fairly definite interval of time following the injection of the toxic dose.

The above procedure was repeated on twelve animals with uniformly negative results as shown in table 1; in no case were there any symptoms of anaphylactic shock. Nine of the animals were subsequently (after two hours) tested by an intraperitoneal injection of 2.0 cc. horse serum. All of these showed signs of shock in 30 to 35 minutes, thereby indicating that all had been sensitized and that, therefore, the previous negative result of intestinal injection was not due to failure of proper sensitization.

These negative results were not considered as conflicting with those of other investigators but rather as confirming the view of Hammersten that absorption of undigested protein takes place only under certain

TABLE 1

NUMBER	INTRAPERITONEAL SENSITIZING DOSE	INTESTINAL TOXIC DOSE	ANAPHYLACTIC SYMPTOMS	SUBSEQUENT INTRAPERITONEAL TEST
	cc.	*cc.*		
1	0.01	2.0	Negative	No test
2	0.01	2.0	Negative	No test
3	0.01	2.0	Negative	No test
4	0.01	2.0	Negative	Positive
5	0.01	2.0	Negative	Positive
6	0.01	2.0	Negative	Positive
7	0.01	2.0	Negative	Positive
8	0.01	2.0	Negative	Positive
9	0.01	2.0	Negative	Positive
10	0.01	2.0	Negative	Positive
11	0.01	2.0	Negative	Positive
12	0.01	2.0	Negative	Positive

unusual conditions—conditions apparently unfulfilled in our experiment. Hammersten (1909) mentions three conditions: *a*, extraordinary permeability of the intestinal walls as in the newborn; *b*, lessened activity of the digestive enzymes; and *c*, flooding a portion of the alimentary tract with protein.

B. *Intestinal injection of large amounts of horse serum.* The condition of flooding a part of the alimentary tract has been frequently fulfilled in the procedure of other investigators. We have tried this by directly injecting from 5.0 to 10.0 cc. horse serum into the intestine of two previously sensitized guinea pigs. The dose was large enough to fill and distend a considerable portion of the intestine at first, although later there was a distribution of the serum throughout the tract and no longer any distention. The animals, however, showed only mild signs of anaphylactic shock, namely, hyperactivity, pawing at the mouth and rapid chewing

movements after an interval of thirty-five to sixty minutes. These mild symptoms lasted only a few minutes. An hour after they disappeared the animals received 2.0 cc. horse serum intraperitoneally, whereupon they manifested marked symptoms within five minutes.

These results are interpreted as indicating that despite the flooding of the intestine, either only a very small part was absorbed in unchanged form or the absorption into the circulation was too slow to produce marked anaphylactic intoxication.

The above procedure of flooding the intestine was not considered promising of further results and it seemed rather preferable to attempt to find the conditions under which the usual small dose of 2.0 cc. horse serum would be sufficiently absorbed in an undigested condition to produce symptoms of shock.

C. *Oral administration of small amounts of horse serum.* The first effort in this direction consisted in the oral administration of the toxic dose. Two previously sensitized guinea pigs were given 2.0 cc. horse serum by stomach tube. Promptly thirty minutes later the animals showed all signs of anaphylactic shock. The inevitable conclusion would seem to be that a sufficient amount of serum protein was absorbed into the circulation. It is impossible to tell whether the absorption took place from the stomach or from the intestine after passage of the serum through the pyloric sphincter. The latter alternative would be surprising in view of the previously observed failure of absorption of the same dose by direct intestinal injection. A possible explanation thought of was that in the stomach there was a temporary stasis of the serum and perhaps pressure upon it due to the normal motility of the organ, whereas in the intestine there was practically no stasis and but little pressure. This explanation suggested another trial of absorption from the intestine, this time under conditions of artificial stasis and increased pressure such as can be produced by ligating off a loop of the intestine.

D. *Injection of small amounts of horse serum into a ligated loop of intestine.* The procedure was the same as for the first set of experiments except that 1.0 to 2.0 cc. horse serum were injected into a loop of intestine, approximately 10 cm. in length, ligated at both ends. It was observed that peristaltic waves frequently passed over the intestine including the ligated portion. As before, the loop was replaced in the abdomen, the wound sutured and the animal allowed to come out of the anesthetic. This procedure was repeated on seven animals. In three of these the loop of intestine was first washed out with physiological saline solution before injecting the horse serum. The food and also the pancreatic juice thus washed out seemed, however, in no way to affect the result, for all the animals uniformly showed the usual symptoms of anaphylactic shock in thirty to thirty-five minutes.

Evidently a sufficient amount of undigested serum protein was absorbed from the intestine under the given conditions of stasis and assumed increased pressure due to peristalsis. These two conditions were not fully differentiated although work in that direction is now in progress and will be reported later.

E. *Intestinal injection of small amounts of horse serum mixed with sodium fluoride.* Having demonstrated absorption from a ligated loop of intestine and failure of absorption from the unligated intestine, the next step was an attempt to so modify the intestinal wall that absorption would take place also from the unligated gut. For this purpose sodium fluoride is commonly used. A small amount was mixed with the usual dose of 2.0

TABLE 2

NUMBER	INTRAPERITONEAL SENSITIZING DOSE	INTESTINAL TOXIC DOSE	ANAPHYLACTIC SYMPTOMS
	cc.	*cc.*	
1	0.01	1.0	Positive
2	0.01	1.0	Positive
3	0.01	1.5	Positive
4	0.01	1.5	Positive
5	0.01	2.0	Positive
6	0.01	2.0	Positive
7	0.01	2.0	Positive

TABLE 3

NUMBER	INTRAPERITONEAL SENSITIZING DOSE	INTESTINAL TOXIC DOSE PLUS 0.06 GM. NaFl	ANAPHYLACTIC SYMPTOMS
	cc.	*cc.*	
1	0.01	1.0	Positive
2	0.01	1.5	Positive
3	0.01	2.0	Positive
4	0.01	2.0	Positive

cc. horse serum and injected directly into the unligated intestine of previously sensitized guinea pigs. Symptoms of fluoride poisoning are apt to supervene and interfere with or mask the anaphylactic symptoms if the dose is too large. It was determined that 0.06 gram of sodium fluoride was just insufficient to produce interfering symptoms of fluoride poisoning. The procedure was repeated on four animals with clearly positive results, the animals showing anaphylactic shock in twenty to thirty-five minutes.

After the animals had shown the signs of shock they were killed and the intestines examined. There were marked signs of congestion but no strictures or other evidences that might lead one to suspect a stasis of the serum in the gut. The assumption was therefore made that the fluoride

so increased the permeability of the intestinal wall that sufficiently rapid absorption of undigested protein was made possible even without stasis.

F. *Sensitization via a clamped loop of intestine.* In connection with the above experiments in which anaphylactic intoxication was produced via the alimentary tract mention may here be made of a number of attempts of the reverse process, i.e., of sensitization via the intestine. The previous experiments indicated that stasis of the serum in the intestine is a condition greatly facilitating the absorption of undigested protein. For this reason sensitization was also attempted under conditions of stasis. Another factor considered was the size of the dose. Obviously the sensitizing dose could not be determined directly, inasmuch as the amount of the serum actually finding its way into the circulation after absorption from a ligated loop was not known. But this amount actually reaching the circulation is the true sensitizing dose and for convenience should not be too large,

TABLE 4

NUMBER	INTESTINAL SENSITIZING DOSE	INTRAPERITONEAL TOXIC DOSE	ANAPHYLACTIC SYMPTOMS
	cc.	*cc.*	
1	0.01	2.0	Negative
2	0.10	2.0	Negative
3	0.30	2.0	Negative
4	0.50	2.0	Positive
5	0.50	2.0	Positive
6	0.70	2.0	Positive
7	0.70	2.0	Positive
8	1.00	2.0	Positive
9	1.00	2.0	Positive
10	1.50	2.0	Positive

otherwise a relatively enormous toxic dose would be required. On the basis of such considerations the following procedure was adopted.

A guinea pig was anesthetized, a 2 cm. incision made through the abdomen and a loop of small intestine drawn forth. Instead of ligating this loop two small bull-dog clamps were placed on it 10 cm. apart. Into this temporarily isolated loop 0.01 cc. horse serum plus 1.99 cc. water were injected close to one of the clamps and then a third clamp placed on the point of injection to avoid possible leakage. The loop was now replaced in the abdomen, the clamps protruding from the incision. After the lapse of thirty minutes the clamps were removed, the incision sutured and the animal allowed to come out of the anesthetic. Three weeks later the usual 2.0 cc. toxic dose of horse serum was given intraperitoneally and the animal observed for signs of anaphylactic shock. This procedure was repeated on a series of guinea pigs using increasingly large sensitizing doses, ranging from 0.01 cc. to 1.50 cc., each diluted to 2.0 cc.

As indicated in table 4, the animals that received the smaller dose, from 0.01 to 0.30 cc., were not sensitized whereas those which received a larger dose, from 0.50 to 1.50 cc., were sensitized as shown by positive symptoms of anaphylactic shock in thirty to thirty-five minutes upon subsequent injection of the toxic dose of horse serum.

Evidently there was a sufficient and sufficiently rapid absorption of undigested protein for sensitization provided the dose was not less than 0.50 cc. Clearly this dose can be considered minimal only for the given conditions, namely, temporary stasis of thirty minutes in a loop of 10 cm. length into which 2.0 cc. of total fluid are injected.

Discussion. The result common to the preceding experiments is that under otherwise normal conditions of the alimentary tract an adequate absorption of undigested horse serum takes place most readily if the serum is in a condition of stasis. But in this condition active peristalsis of the part can be directly observed or assumed. Mechanically considered, the effect of the peristalsis must be an unusual increase of pressure within the intestine and possibly also the stomach. We have been led to infer that such an increase of pressure is the essential factor for the absorption, and sufficiently rapid absorption, of relatively large amounts of undigested protein.

Obviously the next and most important step in the investigation is to measure the intra-intestinal pressure. Work of this nature is in progress. However, even without such direct evidence we believe to have a justifiable assumption and one which promises to be of interest in that it reintroduces a factor in the process of absorption long ago postulated by Voit and Bauer (1869), thoroughly demonstrated by Leubuscher (1885) and by Hamburger (1904) and denied or neglected by Cohnheim (1899), Reid (1901) and others. It is true, the primary interest of the early investigators lay in supporting or refuting a "mechanical" or a "vital" theory of absorption. Nevertheless their work demonstrated a number of important facts concerning the process of absorption. Leubuscher (1885) found that absorption (of saline or serum) increased with increasing intra-intestinal pressure up to a certain optimum (80 to 140 mm. Hg) and then decreased. Hamburger, under more controlled conditions (preventing expansion of the intestine) also found that increased intra-intestinal pressure facilitated absorption and that when this pressure was reduced to zero or to a slightly negative value there was a complete arrest of absorption. Hamburger further states that in the living animal this pressure may be increased by breathing, by peristalsis and by the weight of the intestine.

Leubuscher and Hamburger were concerned in their experiments merely with absorption in the sense of disappearance from the lumen of the gut and not with the condition in which such substances reached the blood

stream. We now know that considerable protein digestion may and does take place during the passage through the wall of the intestine. But this does not influence their finding that variations in intra-intestinal pressure can affect the *rate* of absorption. Accepting their conclusion we wish to add, on the basis of our own findings, that under unusual conditions of increased intra-intestinal pressure proteins are not only absorbed more rapidly but a certain portion actually reaches the blood stream in undigested form; and this portion may be large enough and the absorption rapid enough for sensitization of a normal animal or for intoxication of a previously sensitized animal.

SUMMARY

1. Guinea pigs previously sensitized to horse serum showed no signs of anaphylactic shock upon the direct intestinal injection of small amounts of horse serum (1.0 to 2.0 cc.); but all the usual symptoms of anaphylaxis were manifested after *a*, the direct injection of small amounts into a ligated loop of small intestine; *b*, the direct injection into the unligated intestine of very large amounts (10.0 cc.); *c*, the oral administration of small amounts; *d*, the direct injection into the unligated intestine of small amounts of the serum mixed with sodium fluoride.

2. Guinea pigs were also sensitized to horse serum by direct injection of the serum into a temporarily clamped loop of small intestine, provided the dose used was not less than 0.50 cc.

3. The experimental evidence supports the assumption that an unusual increase of intra-intestinal pressure (as a result of stasis) is the essential factor in the absorption and sufficiently rapid absorption of an amount of undigested horse serum adequate to cause symptoms of anaphylactic shock in previously sensitized guinea pigs or to sensitize normal guinea pigs provided the dose is suitably adjusted.

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THE ENDURANCE OF PRESSURE BY NERVE CELLS AND BY INTERSTITIAL CELLS

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Probably no other single factor has been the cause of greater confusion in the study of the innervation of the digestive tube than the question as to the nature of interstitial cells. By some investigators they have been considered neurones, by others connective tissue elements. A brief outline will illustrate the history of the controversy.

Ramón y Cajal (1889), (1892), (1893), (1909) observed these cells in the wall of the digestive tube and interpreted them as free neurones. To them he applied the term "interstitial cells." Dogiel (1895), (1899), noting their wide distribution and unfailing association with connective tissue, came to the conclusion that they were connective tissue cells. In a paper published in 1896 he reported the presence of two types of neurones, which he designated as type I and type II. The similarity of the type II neurones to the considerably smaller interstitial cells led Erik Müller (1921) to identify one with the other.

In silver impregnations interstitial cells certainly resemble neurones in general form. The processes of these cells ramify into the surrounding muscle layers, a feature highly suggestive of nervous nature. With vital methylene blue both neurones and interstitial cells may be made to stain readily.

The use of methylene blue, however, brings out a number of differences, both in the structure and in the reactions of these cells to the vital dye. In the case of neurones the nucleus is distinct; it is more heavily stained than the cytoplasm, and is separated from it by an hyaline zone (Dogiel, 1895; 1899). Nissl bodies are usually clearly seen. There is a clear distinction between dendrites and axone (Dogiel, 1895; 1899). The axone of a neurone has been seen to terminate on another nerve cell (Kuntz, 1922).

In the same preparations interstitial cells may also be found. In a typical case the following characteristics may be noted. The nucleus is indistinct, that is, the nucleus and cytoplasm appear to stain with about equal intensity; there is no hyaline zone. Nissl bodies are not found; instead there can be seen large numbers of heavily stained granules closely packed in the cytoplasm. The processes of the cell cannot be classified

into dendrites and axone, but appear to be of similar nature. Although they ramify in all directions, often making connections with other interstitial cells, they have never been reported in fusion, or in synaptic relation with neurones.

Although a great majority of investigators probably look upon interstitial cells as connective tissue elements, that opinion is not universal. For example, Erik Müller (1921), while recognizing the considerable differences between undoubted neurones and interstitial cells, nevertheless considers that both are truly nervous. He maintains that there are two types of nerve cells present in the layers of the mammalian digestive tube. Upon that assumption he has built up a most ingenious hypothesis to account for the movements of the digestive tube.

Procedure. Since the purely histological investigations of this problem have given data that are open to more than one interpretation, it seemed advisable to turn to physiological methods in the attempt to secure evidence that might be conclusive.

A frog was pithed, the body cavity cut open, and the intestine clamped here and there between pieces of glass. The entire animal was immediately placed in Ringer's solution for a period of six hours. The clamps were then removed, the intestine excised and put for one hour in a 1:4000 solution of vital methylene blue in physiological salt solution. At the end of that time the intestine was slit lengthwise, and spread out flat between glass slides for examination under the low power of the microscope. The results were striking and significant.

The mucosa and submucosa had been at least partially destroyed by the pressure applied, but the serosa and muscle tunics were left intact. Interstitial cells appeared at all levels, in all parts of the tube, clamped and unclamped portions alike. Undoubted nerve cells, on the other hand, appeared only in the region of the myenteric plexus of the unclamped areas. In the clamped regions muscle cells appeared, but somewhat damaged. In unclamped regions they appeared normal. It must be emphasized that all parts of the tissue appeared to have undergone selective staining; there was no evidence of a general staining such as is secured with the use of methylene blue on killed and fixed tissues. Several frogs were treated in the manner described above and the results were so nearly alike that a description of the findings in one case will suffice for all.

Discussion. The procedure described in this paper was suggested by the work of Cannon and Burket (1913). It will be recalled that they found that the cat's intestine, clamped for three and a half hours, failed to show normal nerve cells in the clamped regions, when, several days later, the cat was killed and the tissues of the intestine fixed, stained and examined. Evidently the pressure and resulting anemia had brought about the death and disintegration of the nerve cells in the myenteric plexus.

That a selective nerve stain can be secured with methylene blue only when the tissues are fresh, is well known. According to Chambers (1923), nuclear staining results just at the death of the cell. Since nerve cells have a high metabolic rate, it is a safe assumption that they would be among the first to succumb to the clamping procedure. Their staining capacity, too, would soon be lost. It is likely that in six hours considerable disintegration would occur. Interstitial cells, on the other hand, were able to endure prolonged clamping, as evidenced by their continued capacity for staining. It is evident that in the case of interstitial cells we are dealing with elements of a nature quite different from that of neurones. If, as appears to be the case, the capacity for staining in a selective way is dependent upon the dying condition of the cell, it is apparent that interstitial cells are able to survive an astonishing duration of pressure and anemia. This endurance must be a reciprocal of their metabolic rate. It is difficult to think of such cells as constituting units in a system requiring the greatest activity. The conclusion seems warranted that they are not nerve cells and do not function as conducting elements. Structurally and physiologically they differ widely from nerve cells. Their wide distribution, unfailing association with connective tissue, and the interweaving of their processes among muscle cells, all suggest a supporting function. The evidence seems to indicate clearly that they are connective tissue elements.

SUMMARY

1. The controversy regarding the nature of the interstitial cells of Cajal is outlined.

2. Interstitial cells and neurones are compared as to structure and distribution.

3. The effects of prolonged clamping upon the staining capacities of interstitial cells and neurones is reported. Results show that the two belong to widely different categories.

4. A study of the distribution, structure and reactions of interstitial cells points clearly to their supporting nature. That they are connective tissue elements seems to be definitely demonstrated.

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CHANGES IN BLOOD PRESSURE DURING SLEEP AS DETERMINED BY THE ERLANGER METHOD

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The onset of sleep, according to the vasomotor theory, is due to the fatigue of the vasomotor center, with a resultant diminution of cerebral circulation. Howell (1897) has been the chief exponent of this theory although he admits (1921) that evidence subsequent to his original experimentation has failed to confirm his position. Shepard (1914), on the basis of his study of cerebral circulation during sleep, states that the vasomotor change, whether it be an increase or decrease of circulation, can be just as well thought of either as a consequence of sleep, or as a cause of sleep. Kleitman (1923) was unable to find loss of vasomotor tone even after 115 hours of wakefulness. He did find a subnormal blood pressure which he attributed in part to a slowed heart, and in part to the muscular relaxation when in prone position. The crux of the matter is that there are circulatory changes on going to sleep and on awakening but, as Shepard says, we do not know whether these changes are causative or resultant of sleep.

The present study offers an analysis of the circulatory changes, as afforded by tracings of blood pressure obtained by the Erlanger (1904) method, which have some bearing on the problem.

It was found that several subjects (young male graduate students) were able to go to sleep while the blood pressure compression cuffs were inflated and that deflation and reinflation of the cuffs did not awaken the subject. The technique consisted of placing a compression cuff around the thigh just above either knee, with a third cuff on the arm just above the elbow. The two leg bands were so arranged that either might be connected through an Erlanger capsule to a tambour carrying a recording pen. The arm band was used with a bracelet stethoscope so that readings of pressure could be taken directly. Respiration tracings were made by means of a Somner pneumograph and a Marey tambour. The subject went to sleep either sitting erect in a chair or lying on a cot.

Two types of curves were obtained during the going-to-sleep period.

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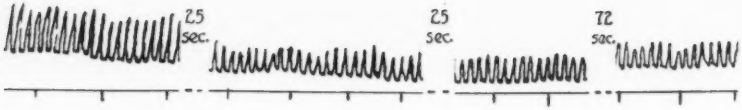

Fig.1

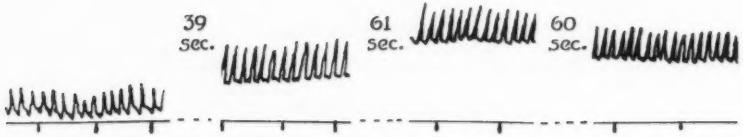

Fig.2

Fig.3

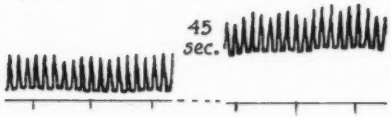

Fig.4

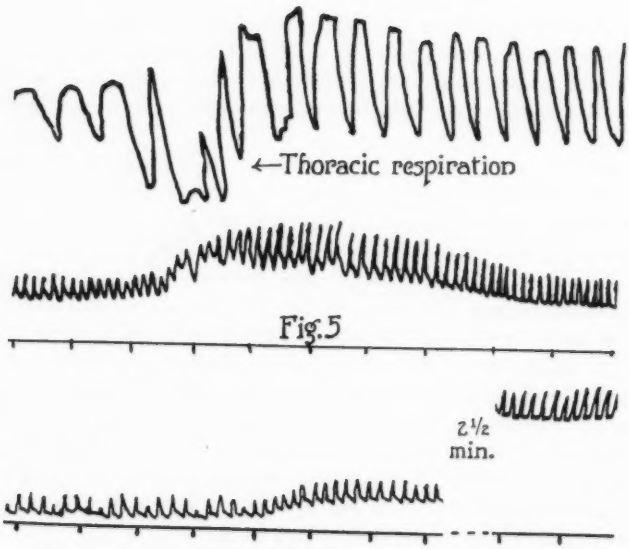

Fig.5

Fig.6

(———5 second intervals)

The first type was obtained when the subject had been awake for some time and then went to sleep. The second type occurred when the subject had been awakened from sleep and was then allowed to go to sleep again. Figure 1 give typical selections from a curve of the first variety. It will be noticed that in the first portion the pulse shows full and rapid (pulse rate, 96; pressure 108 mm./74 mm.). Then during a twenty-five second interval both systolic and diastolic pressures drop to 98 mm./70 mm. During this period the pulse rate dropped to 82 per minute which rate it maintained until the subject was awakened. In the third section of figure 1 the pressure was 94 mm./68 mm. In the last section, 72 seconds later, the pressure was 98 mm./70 mm. which figure was maintained until the subject was awakened. (The pressures, as 98 mm./70 mm., were obtained from the arm using the ausculatory method.) This tracing together with others similar to it fail to show any particular rhythms during this period.

Figure 2 gives sections from a second type of curve which was obtained when the subject went to sleep. In this case a pulse rate of 72 was maintained throughout. Blood pressure at the point of the first section shown was 90 mm./70 mm. Thirty-nine seconds later it was 98 mm./74 mm.; sixty-one seconds later it was 106 mm./78 mm. Following which, it dropped to the point shown in the last section of this figure, 96 mm./76 mm., which figure was approximated until the subject awoke. In curves of this sort there was a definite rhythm of pressure. The rhythm, in the particular curve from which the sections shown in figure 2 were taken, was a long wave of 16 to 20 heart beats, and hence is not clearly indicated in the illustrated sections. Figure 3 gives a section from a similar curve where the rhythm occurs in five pulsations.

The selected portions shown in figures 1 and 2 are taken from a group of 14 records obtained from three individuals. All of the going-to-sleep records were of one of these two types. As has been said, those records taken while the subject was going to sleep after having been awake for some time are of the form shown in figure 1. Here there is a gradual but constant fall of pulse rate and pulse pressure, and of systolic and diastolic pressure; this fall taking place without the assumption of any definite rhythm of pressure until the subject was asleep. If the subject was awakened after he had been asleep and was then allowed to go to sleep again, the records were of the form of figure 2. This form was marked by a constant pulse rate and a practically constant pulse pressure. There was a gradual rise of both systolic and diastolic pressure followed by a gradual fall of both pressures, the entire process taking three to five minutes. This type was marked by a very definite rhythm of pressure. Whether the subject went to sleep lying on a cot or sitting semi-erect in a chair, had no effect on the form of the curve. Müller (1921) reports that the average systolic blood pressure during sleep, for a number of men whom

he studied was 94 mm. Hg. Each of our subjects gave pressures of 94 mm. ±4 mm. during sleep. There were two criteria which appeared in the curves on the basis of which one could say that the subject had gone to sleep. The first was a drop in systolic pressure to approximately 94 mm. and the second was the appearance of distinctive rhythms in the pressure curves. There is, of course, almost always some form of rhythm in the pressure curves of the normal waking individual. This rhythm alters with the onset of sleep, becoming in most cases shorter and more pronounced. In several instances where the breathing was of the Cheyne-Stokes type there was a double rhythm; the long wave of the respiration rhythm being superimposed on the shorter blood pressure rhythm. The entire matter of blood pressure rhythm and indeed of physiological rhythms in general and their interpretation, is one concerning which little can be found in the experimental literature.

When awakening was normal, i.e., not caused by a sudden loud noise or the like, there was a gradual rise of both systolic and diastolic pressure of the sort shown in figure 4. The rise in pressure in the instance illustrated in figure 4 was from 94 mm./74 mm. to 108 mm./78 mm. during a period of two minutes. Figure 5 shows the effect of the ringing of an alarm clock which awakened the subject. The pressure during sleep was 92 mm./68 mm. During fifteen seconds after the ringing of the bell the pressure rose to 114 mm./54 mm. and 20 seconds later had fallen to 104 mm./64 mm. which figure was maintained while the subject lay quiet. Figure 6 gives portions of a record of awakening of a subject whose respiration during sleep had been of the Cheyne-Stokes type. The pressure while asleep was 88 mm./58 mm. The experimenter clapped his hands suddenly, close to the ear of the subject. The subject awakened and the pressure rose rapidly to 96 mm./60 mm. The pressure continued to rise slowly during the next 2½ minutes at which point (the last portion in figure 6) it was 112 mm./66 mm. which continued while the subject lay quiet.

The results of this study do not offer crucial evidence for or against the vasomotor theory of sleep. They do demonstrate, however, several hitherto undescribed facts. These facts must be included or accounted for in any complete theory of sleep. These facts bear a certain relationship to the vasomotor theory of sleep, as it is usually presented.

An outstanding point is that the decrease or increase of blood pressure is closely concomitant to the loss or regaining of consciousness in the sleep process. This fact holds only with the sleep process proper. That is, sleepiness either before actually starting to go to sleep, or after awakening is not associated with a relatively lower pressure. In terms of Kleitman's (1923) hypothesis, sleep is the "switching off" of certain higher cerebral functional levels. When such levels cease functioning, there is an accompanying fall of blood pressure and the assumption of a new and

more regular or automatic rhythm of pressure waves. The weight of evidence from other studies suggests that such circulatory changes are resultant rather than causative. The fact that bodily position, whether semi-erect or reclining, had no effect on the pressure bears on the point. If the drop in pressure (loss of vasomotor tone) were causitive, then reclining, being a position favorable for loss of tone, should lead to a more pronounced pressure drop than that of the semi-erect position, so being more conducive to sleep. However the pressure and pressure curves were practically the same in either position while the going-to-sleep process was no more difficult in one position than in the other. This offers further evidence to the belief that the sleep process is one of removal of superimposed neural control and a return to a more primitive automaticity of function.

SUMMARY

1. Tracings of blood pressure were taken by the Erlanger method, together with ausculatory readings of systolic and diastolic pressure, while the subject went to sleep, during a short nap, and during the period of awakening.

2. As the subject went to sleep it was found that the pressure gradually dropped from approximately 110 mm./74 mm. to 94 mm./68 mm. Certain of the curves assumed a definite pressure rhythm during this period.

3. During sleep there appeared a more pronounced and more uniform pressure rhythm than when the subject was awake.

4. Awakening, as the result of a sudden stimuli, gave curves where the pressure rose very rapidly from a sleep level of 94 mm./68 mm. to 110 mm./74 mm. or higher. Natural awakening, other than that caused by sudden stimuli, was marked by a more gradual return to the normal waking pressure.

5. Bodily position, whether reclining or semi-erect, had little or no effect on the blood pressure during sleep. This together with other considerations seems to indicate that the change in circulation is resultant from, rather than causative of sleep.

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THERMOMYOGRAMS IN RESPONSE TO NERVE STIMULATION

I. The Initial Rise, as Registered by Einthoven's Galvanometer

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The heat given off by muscle, immediately consequent to a stimulus and during the early stage of rising tension, thus far has been correlated less convincingly with the known chemical events than has the heat given off during the recovery phase. Therefore it was thought worth while to repeat experiments on skeletal muscle with certain modifications in the method and instruments used.

The apparatus adopted consisted first of an Einthoven galvanometer provided with coils and storage battery sufficient to produce any desirable field strength, and following the suggestion of Einthoven (1) and Salomonson (1a), with thread of the lowest practicable resistance. Aluminum threads 80 to 100 mm. in length and 3 to 5 μ in diameter, although of short life, have met this latter requirement. The galvanometer was made by Stoppani & Co. of Berne, who incorporated modifications of the Einthoven original design that were indicated by Dr. George Fahr (1b). The instrument is very heavy on account of the extra heavy coils and the extra heavy iron parts making up the magnet; the total weight is about 216 pounds. The instruments are all set up in a basement room. The galvanometer and the projection lantern are mounted upon a shelf consisting of a slate slab of generous proportions (weight about 420 pounds), and suspended, by a modified Julius method, from heavy wooden joists of the ceiling overhead. Two large vanes project downward from the underside of the shelf into two large vats of heavy oil.[1]

This method of suspension has proved wholly successful for the elimination of the usual mechanical vibrations to which buildings situated in large cities and equipped with many diverse machines are apt to be subjected.

The camera used carries a roll of photographic paper or film 60 meters long by 120 mm. wide, and is mounted upon a separate slate slab (weight

[1] For the heavy petroleum used in these vats we are indebted to the Interocean Oil Refining Company who supplied us with a barrel of it. The oil was rendered sufficiently viscous by the addition of melted paraffin wax.

ca 200 pounds), suspended independently but in the same way as the galvanometer shelf.

The optical system of our instrument together with the distance, 103 cm., from ocular to camera screen, as found by actual measurement permits a magnification of 1560 times. This agrees well with the calculated magnification (1545 times) based upon the characteristics given for objective, ocular, tube-length and projected distance. The beam of light used to illuminate the thread has its source at the crater of a direct current arc burning at 17 amperes when at its greatest desirable intensity.

The galvanometers heretofore used for registering heat production of tissues satisfactorily have never had periods less than 2 seconds. The thread galvanometer as we use it in these experiments had periods often as short as 0.5 second and sometimes as short as 0.1 second.[2] In all cases, although we made no direct test, it was felt certain that the delay of the thread was less than that of the thermopile and probably less than that of the actual rise of temperature in the muscle itself.

The lever used was one of the isometric type, the pull being exerted against a spiral spring.

The thermomyograms here analyzed were obtained in response to maximal single make and break shocks of the induced current, the moment of stimulation being automatically recorded by a delicate electro-magnet signal stylus whose shadow was photographed with the thermal response of the galvanometer thread.

The muscles we employed, not being of uniform cross-section, did not permit the use of the direct method of calibration of thermopile and galvanometer. Therefore also we could not take advantage of a thermopile made by the electroplating method of Wilson.[3] Accordingly, the temperature changes within the muscles as registered by our instruments had to be calculated from the theoretical emf of the thermopile, and from the deflection of the galvanometer to a known difference of potential. Because of this, much care must be taken in the construction of the thermopile so that it will quickly and efficiently convert the generated thermal energy into electrical energy. The wires at the junctions of the thermopile must be touching end to end as closely as possible; and the junctions must be free from all excess of solder or flux, so that they offer the least possible modification in electrical resistance, heat capacity, etc., of the wires themselves.

Heretofore we have made use of the method of Bürker for joining the dissimilar wires together. The chief difficulty of this method is that of

[2] The term period as used here does not include the time for the galvanometer to swing back to null point.

[3] See Proc. Phys. Soc. London, 1920, xxxii, 326. These thermopiles are best used when they can be calibrated directly, on account of chance short circuits, etc.

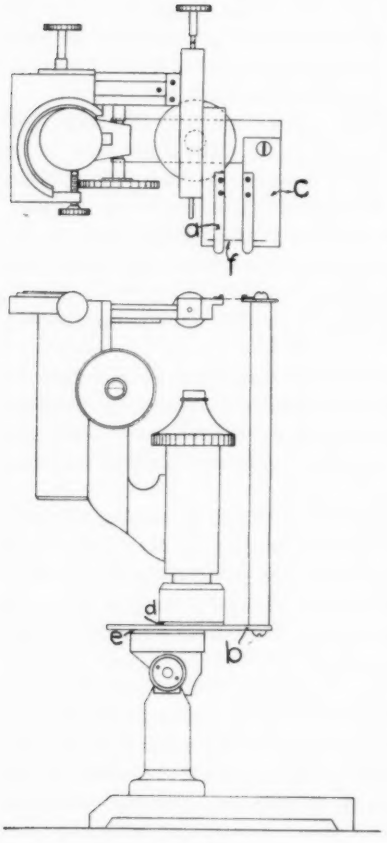

Fig. 1

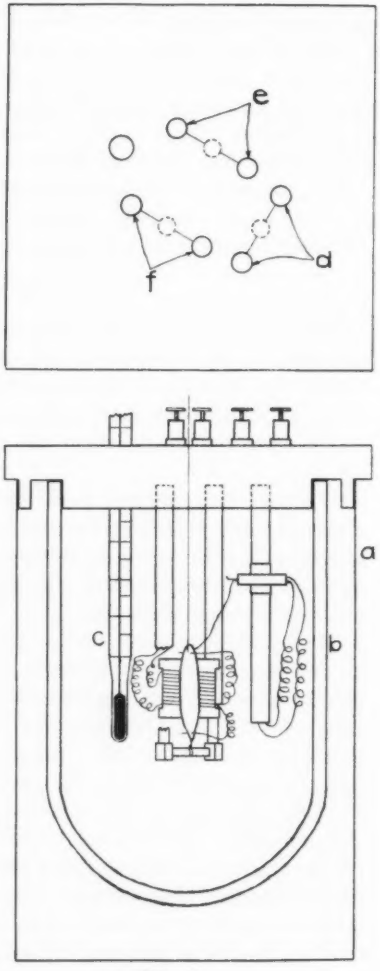

Fig. 2

Fig. 1. Diagram of a three dimensional adjuster for soldering fine wires. See text for description.

Fig. 2. Muscle chamber, a diagram giving verticle section, with cover, projected, showing arrangement of connections:

a, Outer container packed with wool-felt; *b*, Dewar vessel; *c*, mercury thermometer, the part reading 0° to 60° C. extends outside of chamber; *d*, binding posts for current stimulating nerves; *e*, ditto, for stimulating muscles directly; *f*, binding posts leading to thermopile and galvanometer terminals.

getting the wires in exact apposition and of holding them in that position during the soldering process.

To facilitate this procedure the following method was developed and adopted. The lenses, stage and substage were removed from a compound microscope. A mechanical stage was fastened to the top of the body-tube of the microscope (see fig. 1), and a spring clip, *a*, was placed on the moveable arm. A brass plate, *b*, was inserted between the pillar and the arm of the microscope to which was fastened a rod that held a small plate, *c*. Disks of hard rubber, *d* and *e*, were placed in a manner to insulate the body of the microscope from the plate, *b*. This was done in case one wished to weld the wires together with an electrical current, but, so far, this part of the method has been unsuccessful.

The mechanical stage will give motion in two planes, the body tube of the microscope by means of the focusing screws will move in the third plane. Thus by placing the fine wires, *f*, under the spring clips, the wires can be brought in exact apposition, the flux applied to the junction, the junction heated by a small gas flame and the wires soldered, all without the end positions suffering dislocation.

A sixty junction thermopile of iron and constantan wires, the wires 0.12 mm. in diameter, was constructed by this method. The chain of wires when completed was so uniform in cross-section that one could feel only two or three of the junctions when pulling the chain through the fingers. This thermopile was finally used in the present work. It is of the insert type ("Gittersäule" of Bürker) of 30 pairs of junctions, and resistance of 13.3 ohms.

A special potentiometer containing all-copper keys and carefully calibrated shunts and resistances was provided. By a double-throw switch one can connect the galvanometer thread at any moment either with the thermopile circuit or with the standard current circuit. By this arrangement one can add to the record the galvanometer deflection to the calibrating current just before and again at the end of each thermomyogram.

Our method of preparing the threads for the galvanometer from Wollaston wires has not been attended without difficulty. The method is now being improved and a description of it will appear in a later communication.

The thread used for experiments here reported was of aluminum, 79.3 mm. long, 5 micra in diameter, and had a resistance of 148 ohms.

It was decided to use only muscles that could be stimulated through their nerves. The muscle chosen for the experiments of this report was the gastrocnemius of *Rana pipiens*. Stimulation through the nerve obviated (a) electrical disturbances of the galvanometer due to possible slight defects in the insulation of the thermopile, and (b) the heat produced in the muscle by the stimulating current itself just during the early

stage of the muscle contraction, the stage we wish specially to study. Both gastrocnemius muscles of a frog are isolated with their nerves, and the muscles when mounted in the chamber are so placed in longitudinal juxtaposition that their normal inner (tibial) surfaces face each other. The reason for this precaution is the fact that the tibial surfaces of the muscles are the flattest surfaces and therefore make the most uniform contact with all the warm junctions when the thermopile is inserted between them. Indeed the contact is almost as perfect as obtains when the flat sartorius muscle is used.

The muscles are suspended with the tendons at a fixed point below in the chamber, the tibia-fibulas and femurs having been first cut away close to the knee-joints. The latter are then attached together to the cable leading to the muscle lever. This permits one to place the stimulating electrodes *above* muscles and thermopile, and to lead the nerves *up* to the electrodes. This position of the electrodes insures that the heat produced by the stimulating current in the platinum wire electrodes and in the nerve will be dissipated to the upper stratum of air in the chamber, rather than to the stratum immediately surrounding the thermopile. That this precaution is not an excessive one, has been observed by one of us when investigating the heat production of the terrapin's cardia sphincter, and while tetanizing its attached vagus nerve.

The disadvantage of the nerve is that it does not function at 0°C. But by carefully weighing out the ice used in the inner and outer thermostats, levels of temperatures from 3° to 7°C. could be held constant long enough to get tolerably satisfactory series of records.

Results. Biothermal responses have been recorded at least in two instances heretofore using high resistance threads (6). But the galvanometer deflection in those cases was too small to render the measurements satisfactory. With the modifications of the method we have here introduced, a very satisfactory deflection of the thread is obtained as can be seen in the reproduced records, figures 3 and 4.

In our experiments the period of the galvanometer deflection was varied from 0.11 second to 2.5 seconds and the volt sensitivities varied accordingly. Expressed in terms of temperature sensitivities these latter values varied from 6.8×10^{-4} degree C. to 0.8×10^{-4} degree C. per 1 mm. deflection on scale at the camera shutter. All the thermomyograms here considered are responses to single maximal make and break induction shocks, and are typical of all the best experiments.

The speed of the moving photographic paper was not high enough to measure latent periods with any approach to great precision, but in the records of one experiment where the speed of the moving paper was as high as 30 mm. per second we find the following latencies roughly indicated.

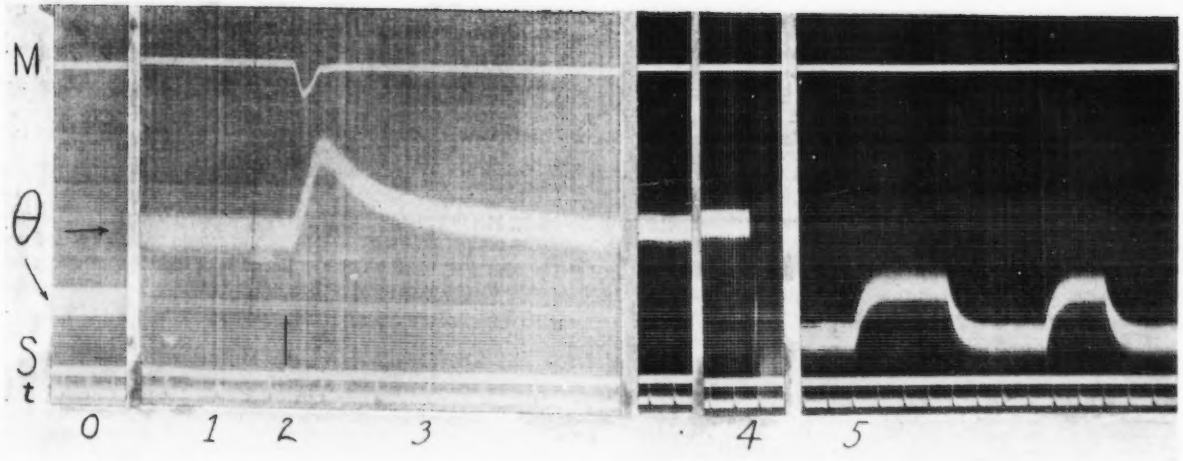

Fig. 3. Reduced one-half. Sections from a continuous thermomyogram of frog's gastrocnemius muscle, from the experiment of vi/2/24, to be read from left to right. The lowest shadowgram, *t*, is that of the time lever in second intervals, the next above is that of the stimulating signal, *S*, an electromagnetic signal in primary circuit of the inductorium. The down-stroke of this lever indicates the make shock that was short-circuited from the nerve circuit; the up-stroke indicates moment of break. The response to this break shock appears in the two shadowgrams above, the middle one, *θ*, being that of the galvanometer thread whose upward deflection indicates a rise of temperature in the muscle during the tension change. This latter is recorded by the uppermost line, the mechanomyogram, *M*, whose down-stroke indicates rise, up-stroke fall, of tension in muscle. Following *θ* in detail: *0* indicates the galvanometer null-point a few seconds before *1*; *1* indicates the resting temperature of the muscles, *r.t.*; *2*, indicates the moment of stimulating the nerves, whereupon the muscles respond with rise of tension and rise of temperature ("initial heat production"); *3*, shows first part of "recovery heat production" and return to *r.t.*, first rapidly, then slowly; *4*, shows the temperature level (recovery heat production) 70 seconds after stimulation (the camera having been stopped 1 minute during the intervening interval), and then switch from the thermopile to shunt circuit of potentiometer; *5*, shows galvanometer null-point and response to calibrating current (3×10^{-6} volt) during the next few seconds. The upward deflection in the original record was 14.0 mm., whence 1 mm. = $3/14 \times 10^{-6}$ V, or $(3/14 \times 10^{-6})/(30 \times 53 \times 10^{-6}) = 14.1 \times 10^{-3}$°C. The deflection of thread at maximum initial temperature rise (at *2*) in the original record was 25 mm., and therefore represents a temperature rise of $25 \times 14.1 \times 10^{-3}$, or 352×10^{-3}°C.; $352 \times 10^{-3} \times 0.83 = 292 \times 10^{-3}$ calorie, the heat produced per gram muscle necessary to raise the temperature the amount observed. (See table 1 under h, number II, 2 b.) The speed of the camera was reduced during the recovery period, hence the marked difference in exposures in the photograph.

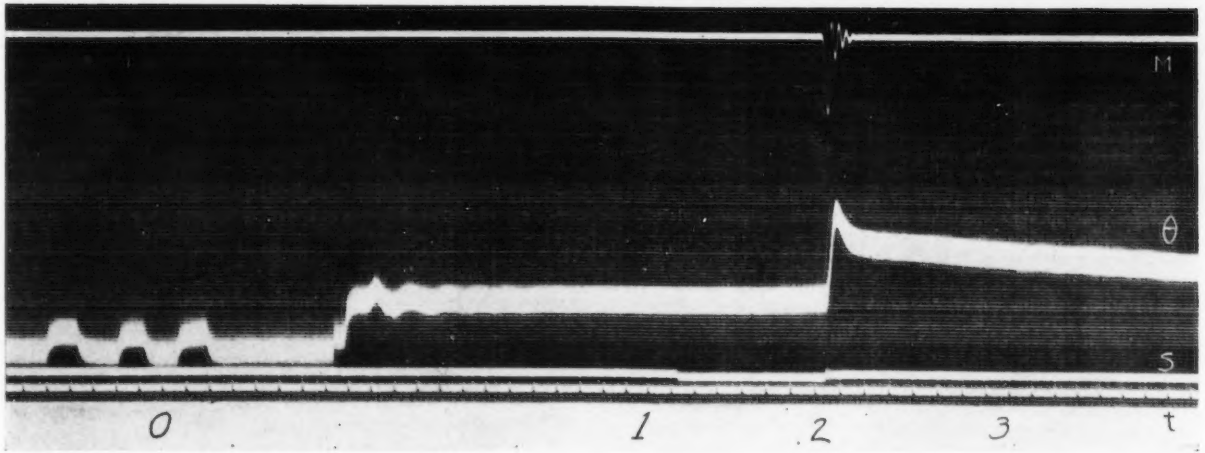

Fig. 4. Thermomyogram from the experiment of V /27 /24, number 4, reduced one-half; the recovery period is cut off. The symbols are the same as, and are explained in legend to figure 3; only the calibrating current, *O*, was thrown in at the beginning of the record. The weight of muscles in this experiment was either not determined, or not recorded; thus the results could not be compared with the others in table 1. This record is here reproduced because it is an exceptionally sharp photograph. Temperature of muscle chamber, 5.6°C. To calibrating current of 3×10^{-6} volt, thread deflects at camera shutter 5.2 mm. in 0.48 second ∴ 1 mm. defl. = 3.6×10^{-4}°C. change of temperature. The muscles are stimulated through their nerves with a single break shock; maximum rise of tension 185 grams, maximum initial rise in temperature, 26 mm. defl., or 936×10^{-5}°C.; if the weight of muscle is put at X then h/t in this case is $\frac{0.83 \times 936 \times 10^{-5}}{185} = 4.2X \times \cdot 10^{-5}$ cal. But, from the other experiments of the series (table 2), the value of h/t for this experiment ought to be 2.85; whence $X = \frac{2.85}{4.2} = 0.68$ grams, the weight of the muscles in this experiment, a not improbable figure.

RECORD NUMBER	MECHANICAL LATENCY	THERMAL LATENCY
	second	*second*
2	0.08	0.113
3	0.10	0.133
6	0.081	0.143

In this experiment the temperature of the muscle-nerve chamber was about 5°C.; the length of nerve that the impulse set up had to travel was nearly 30 mm.; and the resting tension on the muscles varied from 65 to 82 grams. The mechanical latency thus is as great as one would expect under such conditions. It will be noted further that in no case is the latency of the thermal response as short as that of the mechanical response. Indeed careful inspection of all the records, some 50 thermomyograms, convinces one that so far as the galvanometer response is concerned the rise of temperature begins only at the moment or just *after* the rise of tension begins. This corroborates the conclusions of previous observers using more slowly moving galvanometers. A calibrated mechanical (isometric lever) whose shadow accompanies the thermomyograms enables one to read off zero, initial, and maximal tensions maintained or developed during the twitch. The lever was not absolutely isometric, for in the greatest tensions exerted the muscles actually shortened nearly 3 per cent of their lengths.

The thermal responses are calculated from the theoretical performance of the thermopile, the deflections of the galvanometer to a standard current of known potential difference, and the known resistances involved.

In dealing with the data in table 1 we have compared maximum initial heat production, expressed in calories, with maximum tension exerted, expressed in grams, and both for one gram weight of muscle. The pull, that the muscle had to overcome before the writing tip of the isometric lever registered an increase of tension, is set down as "initial tension." This too is expressed in grams per gram muscle. Since the length of the individual muscle, within limits, increases with the increase of this initial tension, we take this figure as a relative expression of the muscle's length. The values of the ratios, initial tension, i, times maximum heat production, h, over the maximum tension, t, exerted, are put in one column under ih/t, and the ratios, heat production over tension, h/t, are also given.

In the experiment of May 29 ten records were obtained, the first two of which were probably taken before conditions were quite stabilized and the last two after some injurious alteration had set in. We therefore are justified in taking averages of only the intervening six records. If so, the average value of h/t is 2.368 with extreme deviations of $+0.292$ and -0.248, or about 11.4 per cent. The variation in heat production and tensions within these several records however is also very slight. The

TABLE 1

	NUMBER OF THERMOMYOGRAM	INITIAL MAXIMUM HEAT PRODUCED PER GRAM MUSCLE (h)	MAXIMUM TENSION PER GRAM MUSCLE (t)	INITIAL TENSION PER GRAM MUSCLE (i)	ih/t	h/t
		calories $\times 10^5$	*grams*	*grams*		
I*	1b	386	212	49.3	89.7	1.82
	2b	425	227	45.3	84.7	1.87
	3m	444	209	41.7	78.5	2.12
	4m	448	209	34.1	73.1	2.14
	5b	510	213	34.1	81.6	2.39
	6b	552	207	31.2	83.3	2.66
	7m	514	212	34.1	82.6	2.42
	8b	526	212	31.2	77.5	2.48
	9b	587	207	34.1	95.6	2.83
	10m	681	218	34.1	106.2	3.12
II	1m	282	85.6	53.0	172	3.25
	2b	292	85.6	44.8	151	3.37
	3b	182	55.8	35.5	115	3.26
III	1b	645	318	21.8	44.2	2.02
	2m	566	318	21.8	38.8	1.78
	3m	585	267	92.1	201	2.19
	4m	566	267	71.3	151	2.12
	5b	510	273	71.3	132	1.86
	6m	526	266	64.3	126	1.97
IV	1	171	33.7	14.0	71	5.08
	2	154	34.2	14.0	63	4.50
	4*	140	29.2	10.1	48.5	4.80
	7	129	29.2	10.1	44.7	4.42
	8	129	29.2	10.1	44.7	4.42
	9	174	28.5	8.4	51.2	6.11
V	1	830	356			2.33
	2	914	466			1.96
	3	772	416			1.86
VI	1	2016	631			3.19
	2	1518	586			2.59
	3	1292	609			2.12

*I. *Expt. of May 29, 1924.* Weight of muscles, 1.318 grams; temperature of muscle chamber, *ca.* 5.9°C.; the subscript letters to the numerals in first column indicate make (m) and break (b) shocks to produce the stimulus. The time between first and last record was two hours and twenty minutes.

II. *Expt. of June 2, 1924.* Weight of muscles, 1.83 grams; temperature of muscle chamber, *ca.* 4.7°C.

III. *Expt. of June 4, 1924.* Weight of muscles, 1.01 grams; temperature of muscle chamber, *ca.* 6.4°C.

IV. *Expt. of May 20, 1924.* All the records are responses to break shock only. Weight of muscles 1.78 grams. Temperature from 1 to 9 inclusive, *ca.* 3.3°C. *The photographs of the missing numbers were defective and could not be used.

V. *Expt. of January 17, 1914.* Weight of muscles, 0.75 gram. Temperature of muscle chamber, at "room temp."

VI. *Expt. of February 16, 1914.* Weight of muscles, 1.15 grams. Temperature of muscle chamber not recorded; room temperature 23°C.

chief significance of the value of h/t is that it serves as an index of the amount of heat produced per gram muscle per gram tension exerted. The absolute quantity is gotten in each case in terms of calories by multiplying value given as h/t by 10^{-5}.

In the experiment of June 2 there are only three records to compare. Here the values of h and t vary widely, with the ratio ih/t showing quite as much variation. The ratio h/t is remarkably constant, the average being 3.29 with the extremes $+0.08$ and -0.04 or a maximal deviation of 2.4 per cent.

The experiment of June 4 rendered us six comparable records. Here the values of h, t and i vary considerably again, but the ratio ih/t varies still more. The ratio h/t on the other hand averages 1.99, $\pm$ 0.205, or with a deviation of about 10.3 per cent to either extreme.

The data under IV (May 20) are from the earliest experiments by our new method. The photographs of several of these records were defective so that one could not read them. Six, however, were successful records and their data are submitted. The average value of h/t here is 4.88 with $+1.23$ and -0.66 as extreme deviations. The variations in h and t here again, however, are not large.

The data in the exhibits V and VI are taken from experiments done a decade previously by one of us (7) using a Paschen galvanometer and a revolving drum for recording the responses. The temperatures in the muscle chambers are not recorded for these experiments, but the whole series was carried out at room temperature. The lever used was of the fret-saw type. It is of interest to note that here the value of h/t is of the same order of magnitude as in the later experiments.

By the above treatment it may seem that the length of the muscles does not receive consideration. Indeed the actual length of the muscles was not observed in these experiments. But the weight was, and among healthy frogs of the same species there can be little doubt that length varies nearly directly with weight. Now this weight factor has been incorporated in our data in two ways. First, in order to obtain the values of h in each case, the observed rise of temperature of the muscles was converted into terms of calories by multiplying by the specific heat of muscle (0.83). By definition this operation gives directly the number h, the calories heat production, per gram muscle. Second, the observed tension exerted by the muscle was also divided by the muscle's weight in each case. The values of h and t, in the table therefore are for one gram weights of muscle and therefore, insofar as weight is indicative of length, h and t do represent values per unit length of muscle. Furthermore, as has been emphasized by others, the structure of the gastrocnemius is such that the length of the whole muscle does not give the length of individual

fibers, therefore, the length of gastrocnemius muscle had we observed it would not have given us the value really wanted.

The tension put on the resting muscle before stimulation is given under i in the table. As will be seen this varied sometimes considerably for the same pair of muscles as well as for different pairs. Whether the value i represents various resting or initial lengths of muscles, or not, the ratio ih/t in our experiments reveals no proportionality.

On the other hand h/t within each or our four experiments varies from the mean, in its extreme value, in each case 11.4, 2.4, 10.3 and 17 per cent respectively. For sartorious muscle A. V. Hill (3, p. 446) finds h/tl approaches a constant, where l is length of muscle and t represents not the whole tension, but the tension exerted over and above that of the resting or initial tension. This ratio in the four pairs of sartorius deviates from the mean values in the extremes as much as 18.7, 45.6, 30.0 and 28.4 per cent respectively. Converting the observed values for sartorius into whole tensions the extreme deviations from the mean value of h/t are 10.8, 33.6, 21.4 and 26.4 per cent respectively. The ratio h/t thus seems to be quite as good for sartorius as h/tl, and surely a better constant for gastrocnemius.

Indeed our ratios of h/t for individual pairs of gastrocnemius as shown in table 1 exhibit a marked tendency to remain constant in each experiment. This agrees with Hartree and Hill (5, p. 144) in their final conclusion on sartorius giving isometric contractions, namely, "that the tension bears a constant relation to the heat production . . . ."; and with Fenn (2) for the same muscle in isotonic contraction, who concludes "increase in energy liberated . . . . is proportional to the tension under which the shortening takes place."

Our value of h/t for the different muscle pairs, however, varies widely,—in the extreme cases more than 100 per cent. It will be noted, however, that the temperature was different in the different experiments. This temperature variation is small, but all of our experience has taught us that small variations in the region between 0° and 7°C. have a great influence generally on such physiological processes as continue to function at all at such low levels of temperature. Inspection of the absolute values of h and t shows that they both diminish with fall of temperature. But it will be noted also that the decrease in tension proceeds more rapidly than the decrease in maximum heat production. The value of the ratio h/t thus increases with fall of temperature. All these facts are brought together in table 2, where the average values of h and t for each experiment are given, large letters being substituted for the corresponding small letters used in the individual experiments; the average temperature appears under θ.

This table summarizes our results most concisely. From it one sees

that, when frog's gastrocnemius is *stimulated through its nerve* with single induced shocks, H and T both increase with rise in temperature. This is just the reverse result from that obtained on frog's semimembranosus (8, p. 408) and frog's sartorius when *stimulated directly* with single or short lasting tetanic shocks (5, p. 136, see column in table under "0.00 second" for values of H; p. 155, see column in table under "shock" for values of T; 4, p. 307 and p. 314). On the other hand our results with single shocks are similar to those gotten by the method of direct stimulation when *tetanic* stimulation of 0.05 second duration or longer was employed (8, p. 411; 5, pp. 136–143, p. 155, p. 156).

This seems to show definitely that for single shocks stimulation through the nerve is much more effective in bringing all the fibers of a muscle into action at once than by application of the electrodes directly to the muscle tissue. The explanation for the inverse relation of H and T to temperature, for single shocks or short lasting tetanus applied directly,

TABLE 2

EXPERIMENT NUMBER	TEMPERATURE OF MUSCLE	TIME OF MECHANICAL RESPONSE (t)	H	T	H/T
	°C.	*second*	*calories* $\times 10^5$	*grams*	*calories* $\times 10^5$
IV	3.3	0.8	149	34.9	4.27
II	4.7	0.6	278	75.6	3.68
I	5.9	0.86	499	210.0	2.37
III	6.4	0.5	578	284.8	2.03
V	?	—	838	412.6	2.03
VI	[23.0]	—	1608	608.6	2.64

may be no other than that the electric current is more or less short-circuited through the saline fluid bathing the outside surfaces of the muscles; the high temperature coefficient of the dissociation of the electrolytes involved would reduce the resistance of this external conductor, probably more than that of the muscle itself, and thus allow still more current to be shunted at higher than at lower temperatures, and thus still more fibers to be left unaffected by the stimulus. There is further the probability that the electric current passing through the parallel-fibered muscle will tend to take the path lying directly between the needle electrodes, the path of least resistance. In this way for a single shock fewer fibers probably are stimulated than when a number of quick shocks, or a short tetanus, is employed.

Whatever the true explanation may be, it seems much more probable that the increases of heat production and tension with decrease of temperature, observed upon semi-membranosus and sartorius muscles, are due to inadequate experimental procedure rather than "the facts (i) that

more energy is liberated by a single shock at a lower temperature and (ii) that a given quantity of energy causes at a lower temperature, a more prolonged contraction" (Hartree and Hill, 5, p. 156). It may not be an easy matter in this sort of experiment to stimulate the thigh muscles of the frog through their nerves, and the question thus may not be settled easily.

But from our results experimenting with the gastrocnemius muscle with its nerve attached it seems more reasonable to assume that the nervous stimulus releases or accelerates a chemical reaction simultaneously in all the muscle fibers, and that this reaction is responsible for the rise in tension primarily; further we may assume that a rise of temperature causes an increase in the velocity of this chemical reaction. Thus finally an increase in the amount of tension developed appears, the latter depending upon the rapidity with which a given change in concentration of one or more of the ions involved in the reaction is effected. For this reason one may conclude rather, *1*, that more energy is liberated by a single shock at higher temperatures, and *2*, that a lower temperature causes a more prolonged contraction in response to a given quantity of energy because the underlying chemical reaction is prolonged.

We may now turn our attention to the ratio of H/T as it appears in table 2. If one compares experiments I to IV inclusive, although the rise in temperature is not large, there nevertheless appears a marked decrease in the value of H/T. However, when with these we compare experiments V and VI where the temperature was surely as much as 23°C. in one case the value of H/T is still nearly the same as it is at 6°C. Since V and VI were done under a different experimental set-up it may not be fair to compare their H/T values with those of the other four experiments. Assuming both sets of experiments are fairly comparable, one must admit that the value of H/T tends to be constant.

A rise in value of H/T with fall of temperature was observed for semimembranosus muscle by Weizäcker (8, p. 409) and also for sartorius muscle by A. V. Hill, but was explained as being due to a faulty tension lever (3, p. 452), one with too much inertia, "overshooting in the more rapid twitches at a higher temperature" (Hartree and Hill, 5, p. 142). Now the lever we used in our experiments has inertia and is not "dead-beat." It may well be that, as the temperature rose, the more rapid movement of the lever caused it to overshoot, thus causing the tension to appear to increase faster than the heat production. One does not find, however, a regular decrease in the time of the mechanical response with increase of temperature in the muscle chamber (table 2, column under t).

By using a proper mechanical lever Hartree and Hill show finally that sartorius muscle of frog when stimulated directly with single shocks or short lasting tetani will give a constant value for the ratio H/Tl, where l represents length of muscle, H the amount of heat, and T the isometric

tension exerted, per gram muscle. The ratio is stated in terms of gm-cm, which when divided by 4.24×10^4 will give its value in terms of calories. From their tabulated results we calculate the average value of H/Tl for shocks and tetani of duration up to and including 0.05 second at 20°C. to be 0.234 gm-cm, or 5.5×10^{-6} calorie; and at 5°C. the average value to be 0.230 gm-cm, or 5.4×10^{-6} calorie (5, p. 143).

From our table 2 it appears that the value of H/T at temperatures 3° to 7°C. is about 31×10^{-6} calories; at room temperature about 23×10^{-6} calorie. Now this is more than four times as much heat as appears for sartorius from the ratio H/Tl. We have shown above why H/T in our experiments needs no further correction for length and that it is most probably as true an expression of heat production per gram muscle per gram tension as can be obtained from gastrocnemius. The difference in absolute values of the ratios in the two muscles therefore can not be due to the factor, l, alone. Nor do we believe that the difference can be due to error arising from our method of heat measurement, as Hill and Hartree would probably maintain (5, p. 134). That our values of H and T agree so well qualitatively (increasing with rise of temperature) as do theirs when they use tetanic stimulation supports our view, and rather points to their method of direct stimulation when single shocks are used, as being the less reliable.

The difference in absolute value of H/T however may well be due in small part to the fact, pointed out by others, that gastrocnemius muscle on account of the diagonal arrangement of its fibers does some work in the "isometric" twitch. As Fenn (2) has shown for sartorius, our muscles then would give off more heat per unit tension than if there were no shortening of fibers at all. Again some part of the differences may also well be due to some fundamental difference in the two muscles (see (2), p. 196 for a discussion of these points).

SUMMARY

1. An improvement in the method of soldering fine-wires for thermopiles has been described.

2. A method suggested by Einthoven has been made practicable for rendering the Einthoven galvanometer microvolt sensitive, and at the same time preserving a period of deflection much more rapid than that of other galvanometers heretofore used for the measurement of heat-production in living tissues.

3. The heat-production of frog gastrocnemius muscle in response to stimulation with single shocks through its nerve has been observed, the responses having been recorded photographically as thermomyograms. From the records so obtained it appears:

a. That although a recording instrument of much less inertia and much

shorter period is used than heretofore for the purpose, the initial rise of muscle temperature is found still to follow rather than precede the initial rise of tension, thus confirming the earlier observation of A. V. Hill on sartorius and of Snyder on heart muscle.

b. That the maximum initial heat production, H, and the maximum tension exerted, T, per gram muscle both *rise with the temperature.* This agrees with the results in sartorius muscle when stimulated *directly* with *tetanic stimuli,* but is *just the reverse of the results obtained on sartorius when stimulated directly with single shocks* (Hartree and Hill).

c. That the absolute value of the initial heat produced per gram muscle per gram tension is from 2 to 3 times 10^{-5} calorie. This is more than four times that observed on sartorius muscle stimulated with single shocks directly.

4. The meaning of the results mentioned in *b* and *c* is briefly discussed in the body of the paper.

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PSEUDO-CRYPTORCHIDISM IN AN ALBINO RAT[1]

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The incidence of cryptorchidism is relatively rare among animals in which the inguinal ring remains open throughout their lifetime. So far as the authors have ascertained from the literature and through inquiries of laboratory workers who have dealt with the rat in their experiments, no case of cryptorchidism or pseudo-cryptorchidism other than that produced experimentally has been reported. For this reason the present case seemed worthy of detailed study. The scope of the investigation includes: 1, study of the sexual behavior and fertility of the male over a period of approximately eight months; 2, repeated measurements of skin temperature for different areas in close relation to the gonads; 3, the condition of the accessory reproductive organs 52 days after removal of the normal testis; and 4, an histological examination of the normal and cryptorchid testes.

Early history of case: Male born February 10, 1924. An inspection of the male when he was approximately 40 days of age led to the discovery that only one testis had descended into the scrotum. There was a small lump in the inguinal region which upon palpation was found to be relatively superficial. One could not ascertain whether it was within the body cavity or between the skin and the abdominal musculature. Gentle massage and pressure applied to this region did not move it. Its contour and size led us to suspect that it was the undescended testis. Thereafter the animal was made the object of special observation and experimentation.

During the spring and summer of 1924 the male was kept in the breeding cages of the colony. He was sexually aggressive and copulated freely. Observations made at this time led to the belief that his sexual behavior was normal and that he was fertile. During the months of November and December special records on the latter point were made. In the course of one week, ten receptive females were put with him. From ten observed matings eight pregnancies resulted. The litters delivered were of average

[1] Certain expenses in this study were met from an appropriation granted by the National Research Council through The Committee for Research on Sex Problems.

size and the young were normal in development. These records assured us that the male was normal with respect to ability to beget young.

Semi-castration: On January 6, 1925, when the male had attained the age of approximately eleven months, semi-castration was performed. The descended testis was removed without injury to the scrotal sac of the opposite side. A portion of this testis was fixed in Bouin's solution for histological study. Fresh samples from both the ductus deferens and the testis examined in normal saline solution revealed an abundant supply of active spermatozoa.

Sexual record subsequent to semi-castration. Three days after semi-castration the tests for fertility were repeated. Four virgin females, one of which was in heat at the time, were put into his cage. Numerous copulations followed and eventually the reproductive tract of the receptive female was closed by a vaginal plug. No spermatozoa were found, however, adhering to samples of the plug removed for microscopic examination. Within a few days the other females came into heat and evidence of copulation was secured. Eight days after the semi-castration two other adult females, both in heat, were put with the male. Copulations were again witnessed and later inspections of the females revealed vaginal plugs. At various times thereafter still other receptive females were placed with him. The total number of different individuals with which he cohabited during a period of 40 days following semicastration was ten. Some of them became receptive several times but no pregnancies resulted. This evidence points to the tentative conclusion that the undescended testis was infertile.

In view of the recent theory (Moore, 1924a, b, c, d) of degeneration in cryptorchid testes caused by excessive temperature in the abdominal cavity, it seemed worth while to make some fairly exact measurements of the skin temperature of this animal. Prior to semi-castration and also just before sacrificing the male several series of measurements were made to compare the skin temperature of the hillock which we suspected to be the undescended testis with the scrotum and with other surface points on the inguinal region.

Temperature-recording apparatus:[2] The thermocouple-galvanometer method was employed. The equipment consisted of two copper-constantan junctions and a Deprez-D' Arsonval moving-coil galvanometer of quick period, essentially as described by Benedict et al. (1919). One of the junctions (known as the standard couple) was located in a thermos bottle so that its temperature could be kept practically constant. The other comparison couple was so mounted that it could be conveniently applied to the skin, either as a bare junction, or held in position by a vulcanized handle with one

[2] This equipment was made possible by the Thomas Welton Stanford Fund for Psychological Research.

side protected by cotton. The junctions were made of fine wire, shaped like a small hair pin, 4 mm. in width, and responded to temperature changes very promptly.

The thermo-couple equipment was standardized each day immediately prior to taking the skin temperature readings. Standardization consisted in placing the comparison junction in a second thermos flask, reading the temperatures of the two baths on thermometers graduated to 0.1°C. and noting the deflection given by the galvanometer. The factors of this particular equipment were such that a difference of one degree Centigrade between the thermo-couples produced a deflection of 140 mm. This was rather more sensitivity than could be used. In order to record the temperatures over a wider range, external resistance was introduced to the amount of 100 ohms, which changed the deflection per degree Centigrade to 60 mm.

Temperature readings were recorded in short series, and the temperature of the standard junction was frequently read. This decreased at the rate of about 0.03°C. each fifteen minutes. In converting the galvanometer deflections into temperature values, the last temperature reading for the standard junction was used.

This type of apparatus is especially adapted to ascertain small temperature differences between adjacent areas. It appealed to the authors as affording an ideal method for taking temperature observations on the rat. The difficulties encountered arise in applying the comparison junction with even pressure and in securing a passive condition on the part of the animal.

Temperature records: The rat was held on a thick towel, in as comfortable a position as possible, exposing the ventral area, in such a way that the body temperature of the person manipulating him would not be communicated to the area concerned. The undescended testis formed a hillock about 10 by 14 mm. protruding from the general contour of the abdominal wall about 6 or 7 mm. On application of the junction there was no change in the position of this testis for it did not slip about, but seemed firmly attached. For purposes of comparison four cutaneous areas were selected: 1, the crest of the hillock representing the skin temperature just over the undescended testis; 2, a position on the opposite side of the abdomen, symmetrical with the hillock and designated as "belly wall;" 3, a position on the groin about half way between the undescended testis and the scrotum; and 4, a well-exposed area on the scrotum.

The position required to make the ventral areas just described easy of access for applying the comparison junction was, of course, not a natural one for the rat. At times he would struggle for brief intervals. We observed that this muscular activity had a marked and almost immediate effect in increasing the skin temperature. During such temperature irregu-

larities it was necessary to interrupt our measurements and wait for quiet periods.

The results for our various series of readings, made on three different dates, are given in table 1, which provides comparisons of temperatures of the different areas defined. The skin temperatures of December 3 in each case represent an average of fourteen readings. Otherwise, in the table, four to six observations were averaged for each point. On December 3, which was prior to semi-castration, the skin temperature of the scrotum overlying the descended testis was one degree colder than for the cryptorchid testis. As the latter area was covered with hair of usual length the actual temperature difference was of course somewhat greater, probably a degree higher, than was recorded. Furthermore the cryptorchid testis,

TABLE 1

Surface temperatures of the testes as compared with other areas for a cryptorchid rat

DATE	ROOM TEMPERATURE	JUNCTION	UNDESCENDED TESTIS	BELLY WALL	GROIN	DESCENDED TESTIS AND SCROTUM
	°C.		°C.	°C.	°C.	°C.
December 3, 1924*.....	19.6	Covered	32.50	33.30	33.00	31.50
February 19, 1925†.....	24.7	Covered	32.68	33.67	33.42	32.68
February 19, 1925‡.....	24.7	Covered	33.44	34.94	33.82	32.75
February 26, 1925.....	21.7	Covered	32.90	34.09		32.23
February 26, 1925.....	21.7	Bare	32.23	33.87		31.50
February 26, 1925.	21.7	Bare	31.34	33.62		31.07

* For this one date, December 3, 1924, each value for skin temperature given in the table is the average of 14 readings; in other cases 4 to 6 observations were averaged.

† About six weeks prior to these measurements of February 19, 1925, the normally descended testis had been removed. The whole scrotum was now much contracted nevertheless temperature readings for that area were taken.

‡ Just before making the second group of temperature readings on February 19, the hair was closely clipped on the areas concerned but no injury was done to the skin.

due to its close proximity to the abdominal cavity, must have a higher average temperature than the skin immediately overlying it. The descended testis is not so protected from heat loss and for this reason more closely approximates the surrounding skin temperature of the scrotum. The difference between the mean temperatures of the two gonads is therefore undoubtedly greater than indicated by our measurements. The hillock, although warmer than the scrotum, was colder than the comparison, hair covered areas of belly wall and groin by 0.8 and 0.5°C. respectively. The cutaneous area overlying the cryptorchid testis therefore represents approximately a mid position between the temperature of the normally distended scrotum and the hair covered belly wall.

About the same type of result was found on comparing the different areas on other dates, i.e., after the normal testis had been removed. On February 19, before the hair was clipped short over the areas concerned, the hillock was 0.99°C. colder than the belly wall and 0.74°C. colder than the groin. It averaged just the same temperature as the scrotum which at this time was of course much contracted. With the hair clipped the temperatures registered higher by 0.76, 1.27 and 0.40°C. for the hillock, belly wall and groin as named. With this changed condition the difference between the hillock and the belly wall was 1.5°C. while the groin was only 0.38°C. warmer than the hillock. The first measurements on February 26th show the hillock 1.19°C. colder than the belly wall, but with the junction applied bare, that is, without the handle which acts as a blanket about the junction, the differences were more prominent, being 1.64 and 2.28°C.

It is obvious from the foregoing skin temperature results on the pseudo-cryptorchid rat that the cutaneous area above the undescended testis was considerably cooler than adjoining areas. How much of this may be due to contour and how much if any to a temperature characteristic for gonadal tissue cannot be assigned.[3] There is no doubt but that the scrotum, especially when naturally distended by one or both testes has a skin temperature that is measurably below that of the abdominal wall above and in the groin. The skin temperature difference for the two testes in this case, taking the influence of the hair into consideration, was probably about two degrees Centigrade.[4]

Necropsy: The abdomen was opened and the skin and superficial fascia over the left hypogastric area were dissected free from the abdominal musculature.[5]

a. Inguinal rings: The left inguinal ring, on the side of the undescended testis, appeared to be normal and resembled that on the right side. It contained the ductus deferens, nerves, blood vessels, and several fatty

[3] It seemed inadvisable to attempt to measure subcutaneous temperatures as we desired to make an histological examination of the cryptorchid testis. Slightly different positions on the hillock made by this testis were tried and seemed with a bare junction to give slight differences of from 0.1 to 0.4°C., but these differences were not nearly as marked as when the junction was placed just off the testis where the temperature would be about one or one and a half degrees higher than on it.

[4] Some additional temperature observations for different areas on the rat were recorded while the animal was sitting in usual posture, quiet, without being held and the junction was applied with the handle. The positions and values were as follows: top of back, 31.72; sacrum, 30.93; base of tail, just beyond hair, 28.12; on tail, 1 cm. distal from base, 27.44°C. More distal positions on the tail were still colder. Such regional differences, with lower values for more distal portions conform with the findings of Benedict, Miles and Johnson (1919) for man.

[5] Autopsy performed with the assistance of Dr. Earl T. Engle, Department of Anatomy, Stanford University.

appendices which are normally found associated with the testis and spermatic cord.

b. Accessory reproductive organs: Prostate and seminal vesicles were turgid with fluid, indicating normal secretory activity. A plug of coagulated seminal fluids was found protruding from the glans penis. Ejaculation usually occurs when mature animals are killed by trauma.[6] In this case the phenomenon furnished additional evidence for normal secretory activity of the accessory reproductive glands and functional ability of the mechanism for ejaculation.

c. Testis: The testis and epididymis supported by the tunics of the former and the dartos muscle had passed through the inguinal ring but instead of reaching the scrotum had been displaced upward into the inguinal region where they lay between the skin and superficial fascia on the one hand and the deep fascia and abdominal musculature on the other. The testis was immobile due to strong adhesions to the abdominal wall, yet it did not appear to be compressed by the surrounding tissues. When all adhesions were removed it could be returned to the scrotum or to the abdominal cavity through the inguinal ring. Its size and weight were about two-thirds those of the normal testis; and, so far as one could tell by inspection, the superficial blood supply was comparable with that of a normal testis.

The epididymis though relatively small was proportionate to the size of the testis. It lacked the milky color so clearly visible in the epididymis of a normal gonad filled with secretions and spermatozoa. Fresh specimens of the testis examined microscopically had no spermatozoa. This sustains the evidence for sterility previously gotten from breeding tests.

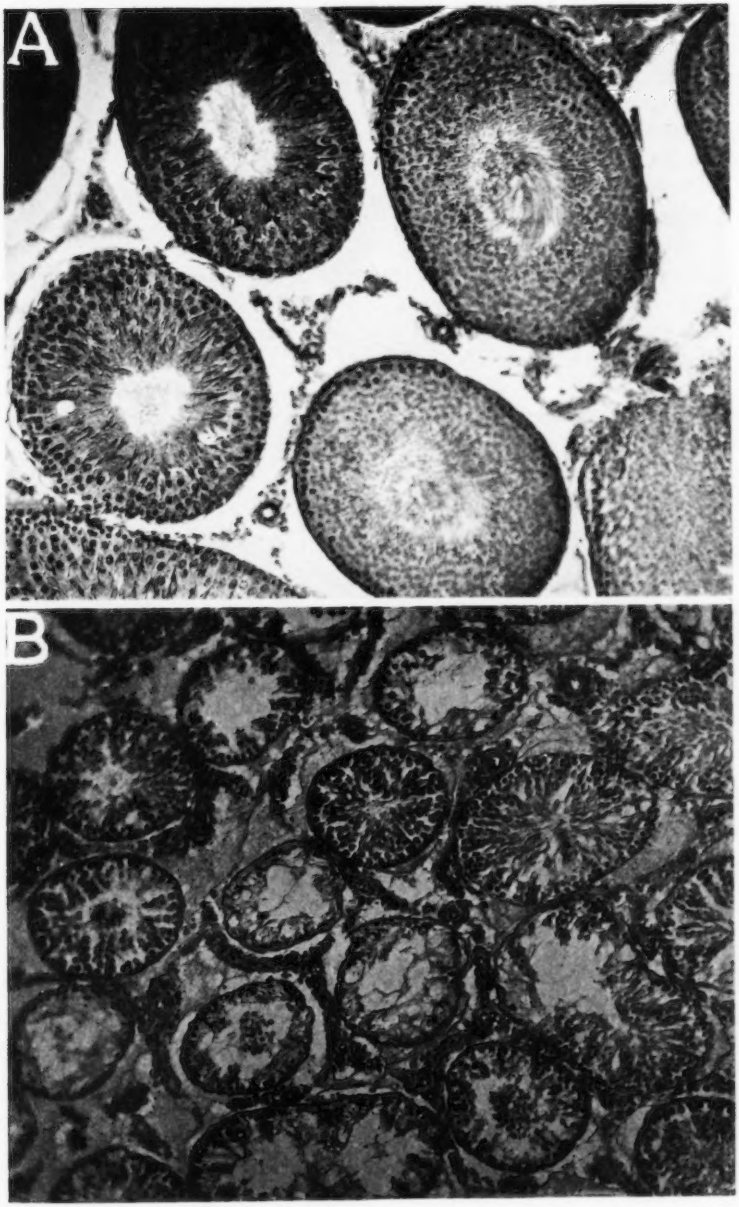
Further evidence pointing to the same conclusions was secured from microscopic examinations of stained specimens of the gonad.[7] Figure 1 is a microphotograph of slides from the cryptorchid, *b*, and the normal, *a*, testis. Both specimens were similarly treated and stained, hence we may assume that the outstanding differences shown in the pictures correspond to the actual differences in the tissues.

The tubules of the cryptorchid testis have atrophied until their diameters

[6] Animal killed by trauma to neck and base of skull.

[7] Fixed in Bouin's fluid. Stained with hematoxylin and eosin.

Fig. 1. Microphotographs of the normal testis, *A*, and the cryptorchid testis, *B*, from the male rat considered in this study. Tissues were similarly treated and photographs made at the same degree of magnification, × 140. Specimen *A* shows all normal stages of spermatogenesis; the lumina of the seminiferous tubules have many mature spermatoza in them. The germinal epithelium of specimen *B* is in a state of degeneration. In it none of the advanced stages of spermatogenesis are to be seen and the testis is sterile.


A
B

are much less than those of the normal testis. In some the germinal epithelium has almost entirely disappeared; in others, only a single layer of cells lining the wall of the tubule are found. For the most part epithelial cells are small and their nuclei pycnotic. In no tubules has spermatogenesis reached the stage of spermatids. On the other hand, in the normal testis myriads of spermatozoa are present in the tubules. The blood supply appears normal in the cryptorchid testis.

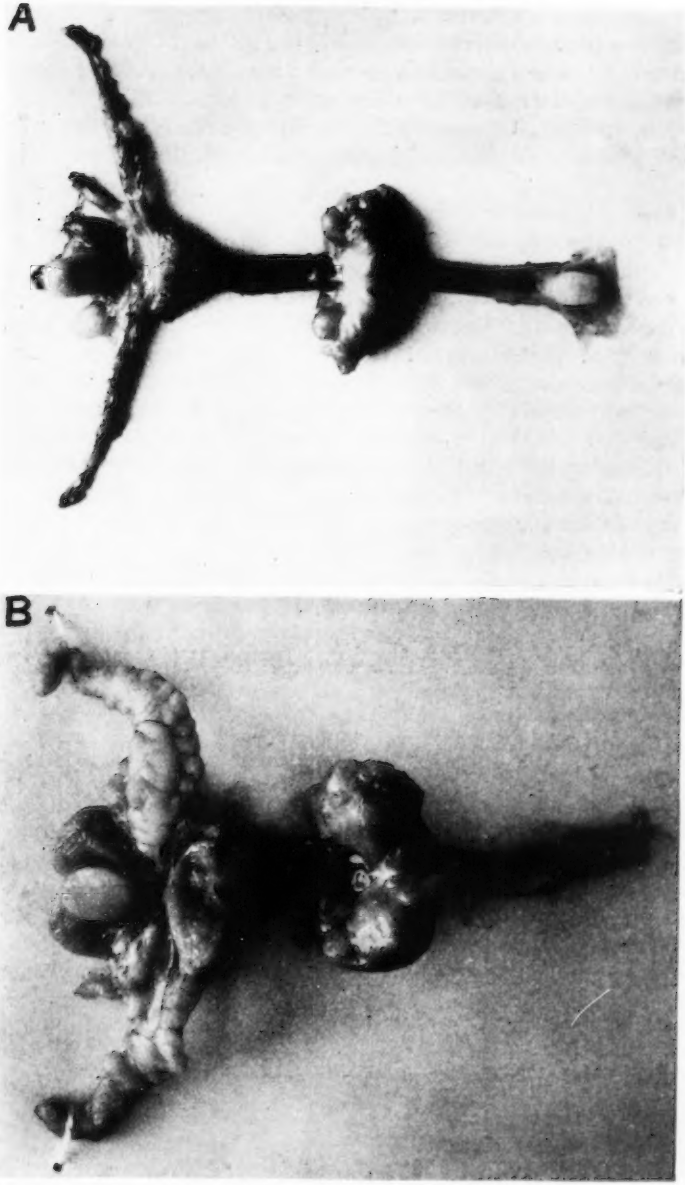
It is worthy of note that the cryptorchid testis in this male closely resembles similar testes described by Moore (1924b) in his studies of experimental cryptorchidism.

The accessory sexual apparatus: The accessory reproductive apparatus of this and another male of approximately the same size, age, and sexual experience are presented in figure 2. The control animal, *A*, lived as a total castrate for a period of time equal to that during which the cryptorchid male lived after semi-castration.

Referring to *B*, the cryptorchid animal, the seminal vesicles, coagulatory glands (Walker) and the prostate are turgid with secretory products. No sign of regressive change is noticeable in any part of the tract. That is not true of the control, *A*, which had been totally castrated for a similar period of time. Reduced secretory activity in the seminal vesicles and coagulatory glands has permitted them to become flaccid and the muscle ischio-cavernosus is much reduced in size. Signs of degeneration are unmistakable. As for lengths of the tracts there was little, if any, difference. From the foregoing evidence we are justified in the conclusion that the sexual hormone was present in the case of *B* and maintained the accessory reproductive apparatus in a normal condition through the period of semi-castration.

Although sexual vigor was retained throughout the period following semi-castration this cannot be added to the evidence that the undescended testis was furnishing the hormone. Many males of this age retain their copulatory ability and sexual vigor after total castration for a much longer period than 45 days.

Fig. 2. Reproductive tracts of the cryptorchid male of this study, *B*, and the tract of a normal male, *A*, of approximately the same age, size, and sexual experience, × 1½. The latter had been totally castrated for a period of time equal to the time the cryptorchid male had been deprived of his normal, descended testis. Atrophy within the tract of *A*, total castration, is very apparent. No atrophy in *B* is apparent.


A
B

SUMMARY

1. A case of pseudo-cryptorchidism in the albino rat was discovered in which the right testis assumed a position between the skin and abdominal musculature of the groin.

2. Tests for fertility conclusively demonstrated that the male was fertile until the normally descended testis was removed. Thereafter he was infertile.

3. Several series of temperature measurements for the skin overlying the cryptorchid testis showed that it was definitely colder than the abdominal wall surrounding it but warmer by approximately two degrees Centigrade than the scrotum containing the normal testis.

4. An histological examination of the cryptorchid gonad showed it to be infertile. Spermatogenesis was arrested in the earlier stages. No germinal epithelium at all was found in some tubules; in others a single layer of cells lined the wall. Degenerative changes were in progress in all tubules.

5. During a period of 52 days no observable degeneration of the accessory sexual apparatus followed removal of the descended testis. The atrophy of these organs in a normal control totally castrated for an equal period of time was very marked.

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ACTIVITY STUDIES ON MALE CASTRATED RATS WITH OVARIAN TRANSPLANTS, AND CORRELATION OF THE ACTIVITY WITH THE HISTOLOGY OF THE GRAFTS

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As shown independently by Wang (1923) and by Slonaker (1925) caged rats given access to a revolving drum show periodic fluctuations in their daily voluntary activity. Every fourth day there is a sudden burst of activity which is in great excess of the activity recorded on each of the three intervening days. These observers were able to correlate this periodic fluctuation in running activity with the ovulation cycle. They found by means of vaginal smears, first applied to the rat by Long and Evans, that the fourth day peak of spontaneous activity coincided with the time of oestrus, and that the three days of lessened activity were synchronous with the three days of the dioestrus. Wang showed that female rats with both ovaries removed were much less active than normal animals (about 90 to 95 per cent less measured by the number of revolutions per day). This low level of activity is maintained throughout the life of spayed rats. A similar decrease in spontaneous activity occurs during pregnancy, lactation, and pseudo-pregnancy produced by mechanical stimulation of the cervix uteri. The low level of activity is present also before sexual maturity, and recurs in the period of senility. In addition to a very marked decrease in spontaneous activity, the ovariectomized, pregnant, pseudo-pregnant, lactating, immature, and senile animals also show a complete disappearance of the characteristic recurrent four-day activity rhythm of normal sexually mature female rats. These several observations led Wang and Slonaker to conclude that the spontaneous activity of the female rat is markedly influenced by the presence of ovaries.

The activity of the male rat is greatly affected by the presence of the testes. In contradistinction to the recurring rhythm of the normal female, the male shows no cyclic rhythm of activity; furthermore, the activity of

[1] The behavior part of these experiments was worked out in the Psychological Laboratory of the Phipps Clinic, Johns Hopkins Hospital by Ging H. Wang and Curt P. Richter. The anatomical and histological study of the grafts, started in the Anatomical Laboratory of the Johns Hopkins Medical School and completed in the Department of Anatomy of the University of Rochester, was made by Alan F. Guttmacher.

male and female rats may be distinguished by the higher level of activity of the female. The average daily activity of the male varies between two and eight thousand revolutions per day, while that of the female varies between six and twelve thousand revolutions per day. These statements are based on our records obtained from approximately sixty animals during

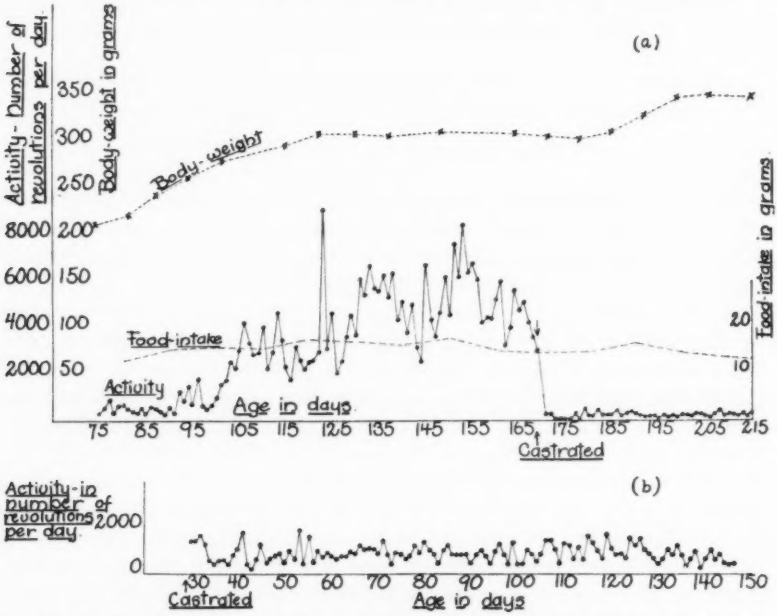

Fig. 1a. Graph showing effect of castration on spontaneous activity of adult rat. The number of revolutions per day in the revolving drum are indicated on the ordinates and the age of the animal in days on the abscissae. This graph also shows the body weight and the average daily food-intake for 10-day periods. This animal was castrated at 169 days. It will be seen that immediately following this operation the activity showed a marked permanent decrease from 4000 to 7000 revolutions per day to a low level of only a few hundred revolutions per day.

Fig. 1b. Graph showing effect of castration on spontaneous activity when animal is castrated at an early age. This animal was castrated at 30 days, just before it was put into the drum. The activity remained on a fairly low level throughout the rest of the animal's life.

the past three years. At the present time it is not possible to give more exact running averages for the two sexes, because of the large individual differences that exist within the two sexes; also because the average running level reached by the adult is dependent on the age at which the rat first learns to run in the drum. We have usually found that the higher running levels are reached by animals that learn to run when they are very

young. After removal of the gonads of the male, the castrate,—like the spayed female,—maintains a constant low level of activity which is 60 to 95 per cent lower than the pre-castration level. The activity of the male castrate is quite similar both in amount and form to that of the spayed female. Our experiments have demonstrated that the testes have an important influence on the spontaneous activity of the male, as is shown in figure 1, a and b.

On the basis of the results obtained from such experiments, it becomes possible to make a quantitative as well as a qualitative study of the effect of transplantation of the sex glands. The flat low level of activity of the castrated male and the spayed female serves as a base line upon which changes in activity following transplantation of the ovaries or the testes may be recorded. Our knowledge of the presence of the four-day activity cycle in the female, and its absence in the male, and the higher running level of the female, makes it possible to recognize definitely signs of male or female behavior.

The following experiments form a study of the effect on behavior of the transplantation of ovaries into previously castrated males. The specific problem involved may be formulated as follows:—How is the low level of the castrated male affected by the transplantation of ovaries, and to what extent are the female characteristics, the four-day cycle and the higher running level, transferred to the castrated male through the grafted ovaries?

METHODS. Activity in these experiments was measured by the methods and technique described by Wang (1923). To obtain quantitative records of periodic activity which has its source within the body, it is essential that environmental conditions be carefully controlled. The room used for the experiment was fitted with double semi-soundproof doors, and situated in a part of the laboratory free from external noises and disturbances. It was kept as clean and well-ventilated as possible, with constant illumination by a dim red light, and with a uniform temperature of 20°C. The animals were disturbed once each day at noon for one half hour, when the cyclometer readings were recorded and the food-cups weighed and refilled; also once each week on Monday afternoon from two to four, when the cages and room were cleaned and the animals weighed.

In behavior work the physical condition of the animal is of utmost importance; for unless one is working with animals in good health, the results become too complicated for analysis. We have found that the food-intake[2] curve, and the body weight records, give us a very simple and

[2] A special diet recommended by Dr. E. V. McCollum was used. We have given this diet to our rats for three years and have had excellent results. The formula with food curves showing the average amount of food eaten by animals of various ages and under various conditions may be found in papers by Wang referred to above.

reliable check on the health of the animals; so that in these experiments as in previous activity experiments, a record was kept of the amount of food eaten every day by each animal (Wang, 1925). Food was left in the cages at all times, and the conditions of obtaining it were kept constant and the same for each animal. It was possible to observe these precautions because each animal was kept in a separate cage. Where there are more animals in the same cage it is usually not possible to keep the food conditions constant.

Twenty-four male rats were used. They were castrated at various ages, from 27 to 90 days after birth. A short time was allowed following castration and before transplantation to be certain that the animals were running consistently on the new low level. The time allowed varied from ten to twenty-five days; but we have since learned that this was an unnecessary precaution. From observations on over forty animals we know that when castration or spaying is performed at an early age or before the animal begins to run a great deal, activity remains on a low level throughout the rest of the animal's life. Castration and spaying after the animals have already reached a high running level causes a similar decrease in activity but not so great.

We used the following technique in transplanting the ovaries. Both the male previously castrated and the female from which the ovaries were to be taken (always a litter mate) were anesthetized. Then the abdominal wall of the female was opened and the ovaries were located and left in a position for quick and easy removal. In the male a mid-line incision was made exposing the recti muscles in one of which a small pouch was prepared for the ovary by separating the exposed muscle fibers into two layers with

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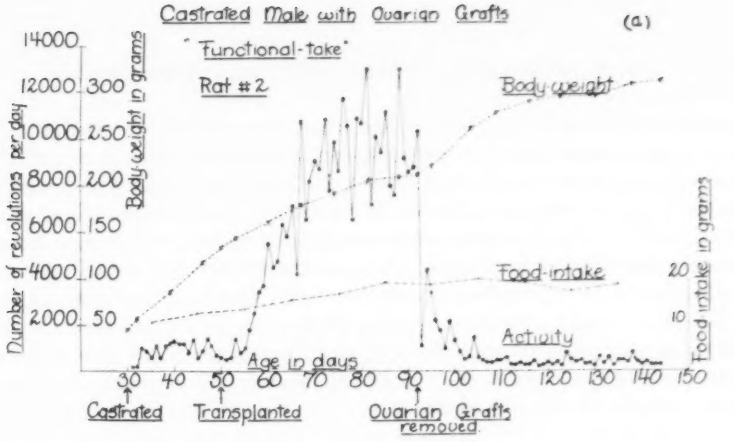
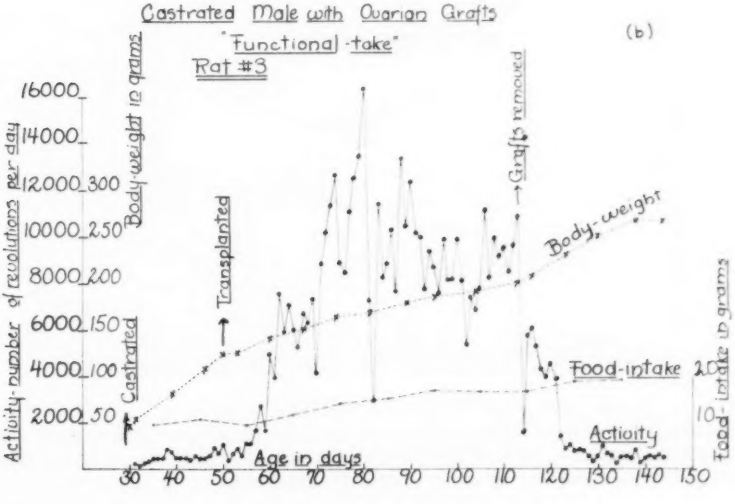
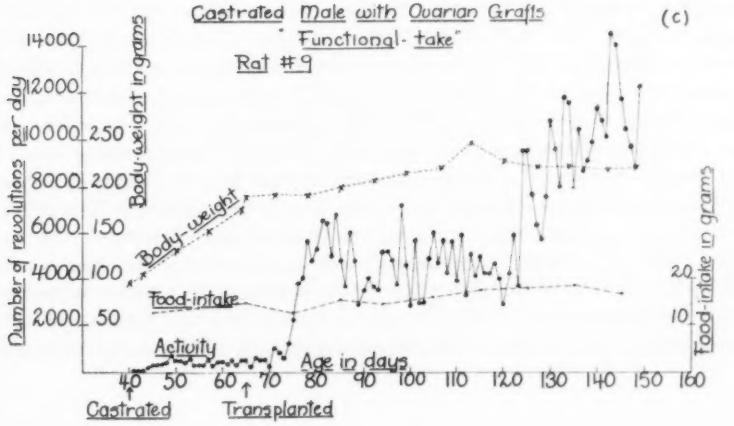
Fig. 2a. Rat 2. Functional take. Record showing the effect of transplantation of ovaries on the activity of a castrated male. Number of revolutions per day on the ordinates, age in days on the abscissae. Age at time of castration, 30 days. Age at time ovaries were transplanted, 50 days. The increase in activity started about 6 days after transplantation and reached a plateau some 20 days later. The grafts were removed when the animal was 93 days old and almost immediately the activity returned to the low pre-transplantation level. Record also shows the body weight of the animals, and food-intake in grams. The marked increase in body weight following removal of the grafts may be noted.

Fig. 2b. Rat 3. Functional take. Legends the same as for rat 2. Animal was castrated at 29 days. The ovaries were transplanted at 50 days. The grafts were removed at 113 days. The retardation in growth following the transplantation of ovaries is shown by the marked flattening of the body weight curve. The record also shows the decrease in activity and the increase in body weight following removal of the grafts.

Fig. 2c. Rat 9. Functional take. Legends the same as for rat 2. Animal was castrated at 30 days. Ovaries were transplanted at 65 days. Record shows the two stages of increased activity following the transplantation. This record also shows a very great flattening of the body weight curve after the transplantation operation.


Castrated Male with Ovarian Grafts
(a)
"Functional-take"
Rat #2
Body weight
Food-intake
Activity
Number of revolutions per day
Body weight in grams
Food intake in grams
Age in days
Castrated
Transplanted
Ovarian Grafts removed


Castrated Male with Ovarian Grafts
"Functional-take"
(b)
Rat #3
Grafts removed
Transplanted
Castrated
Body-weight
Food-intake
Activity
Activity-number of revolutions per day
Body-weight in grams
Food-intake in grams
Age in days


Castrated Male with Ovarian Grafts
(c)
"Functional-take"
Rat #9
Body-weight
Food-intake
Activity
Number of revolutions per day
Body-weight in grams
Food-intake in grams
Age in days
Castrated
Transplanted

a blunt pair of forceps. One of the ovaries of the female was cut off, freed from fat and from the remnants of the Fallopian tube, then cut into five or more pieces which were imbedded in the pouch already prepared for them in the rectus muscle of the male. The incision was closed with silk sutures. With a similar technique the other ovary was transplanted into the opposite rectus muscle. The incisions in the abdominal wall and in the skin of the transplanted castrate were closed with fine silk sutures.

RESULTS. Following the transplantation of ovaries, seventeen out of the twenty-four castrated animals showed an increase in activity. These animals will be referred to as "functional takes." The records of three of these animals which showed the largest increase in activity are shown in figure 2, a, b and c. The number of revolutions per day is given on the ordi-

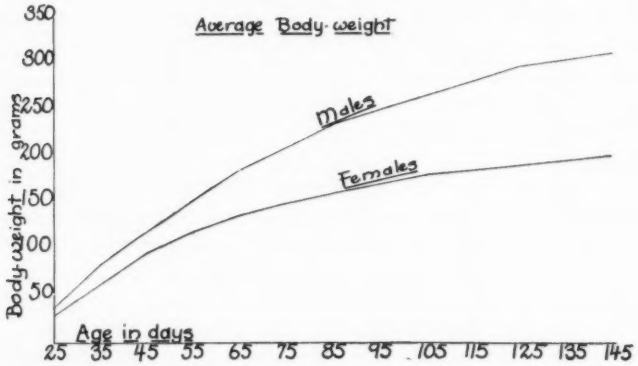


Fig. 3. Normal growth curve of males and females based on records obtained from twenty males and twenty females from our own colony kept under essentially the same conditions as those of the present experiment. Body weight in grams is given on the ordinates and the age in days on the abscissae.

nates and the age in days on the abscissae. The records also show the body weight curve and the daily food-intake in grams. Rat 2, whose record is shown in figure 2a, was castrated at 30 days and was immediately placed in a running cage, where it showed the usual low level of castrated animals. When it was 50 days old ovaries were transplanted into it. After six days a sharp increase in activity occurred which continued steadily for the next twenty days until a plateau was reached at almost eleven thousand revolutions per day. This high level was maintained up to the time of the removal of the grafts when the animal was 93 days old. Following removal of the grafts the activity diminished again (a decrease of 95.7 per cent); so that within a few days after the operation the activity had returned to its pre-transplantation level. It will be noted that the activity of this animal shows the features that are characteristic of the activity of the

female, that is, the presence of the four-day cycle and the high level of activity. Of the six cycles that occurred before the grafts were removed three were four days in length, two five days, and the other three. The level of activity reached, which was almost eleven thousand revolutions per day, was considerably higher than that of the average male rat. Further indications of the effect of the transplantation of ovaries into the castrated males may be seen in the body weight curve of rat 2 in figure 2a. At 90 days, at the time the grafts were removed the weight of this animal was 210 grams, which is considerably less than that of the average male at this age as is shown in comparing it with our average body weight curve of the males and females in our colony (fig. 3). These curves are based on the

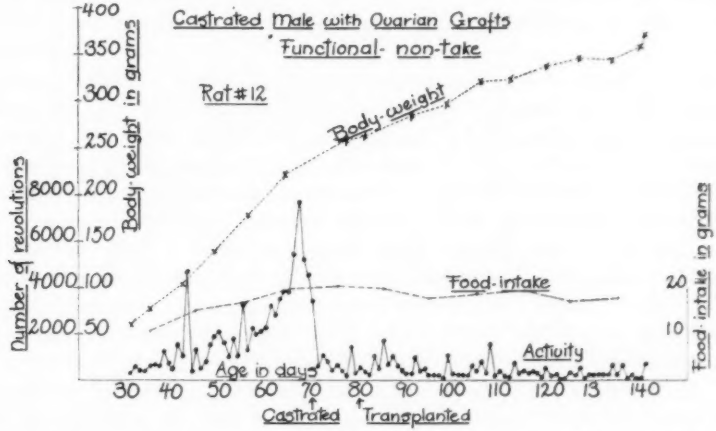


Fig. 4. Rat 12. Functional non-take. Animal was castrated when it was 70 days old. Ovaries were transplanted 10 days later. The transplantation had no apparent effect either on activity or on body weight.

average weekly weights of twenty males and twenty females which were given the same food and kept under approximately the same conditions as the animals reported in this paper. The influence of the ovaries on the body weight is demonstrated more strikingly by the marked increase in body weight following removal of the grafts. At 145 days when the experiment was discontinued, this animal weighed 313 grams which is 8 grams above the average weight of males at this age.

Rat 3 in figure 2b shows a somewhat different record. It was castrated at 29 days and immediately started in the running cage. The transplantation was done on the fiftieth day. The increase in activity began the seventh day following the operation and continued steadily for the next twenty days reaching a plateau 25.6 times higher than the pre-transplantation level. The activity and the cycles are less regular than in

rat 2. The removal of the grafts was followed within five days by a marked drop in activity (94.1 per cent) to the pre-transplantation level. The body weight curve of this animal was flattened very much more by the successful ovarian transplantation than was that of rat 2. Up to the time of transplantation at 50 days, its body weight conformed very closely to that of our average weight curve for the male; but immediately after the transplantation the curve became remarkably flattened. At the age of 113 days, when the grafts were removed, the animal weighed 200 grams which is 70 grams below the weight of males at this age and only 20 grams above the average female weight. The influence of ovaries on the body weight is shown further by the marked increase which took place following removal of the grafts so that within thirty days following this operation the weight of the animal rapidly approached the body weight curve of the male.

Rat 9 in figure 2c following transplantation exhibited a marked increase in activity very similar to that shown by rats 2 and 3, except that in this animal the increase in activity took place in two stages. In the first stage the activity increased for fourteen days when a definite plateau was reached at about 5000 revolutions per day. This plateau was maintained for approximately forty days after which a second increase in activity, more marked than the first, took place. Thus thirty days later a daily average of 10,000 revolutions was reached, with one peak of activity of over 14,000 revolutions. Activity cycles were present in this animal, especially during the second part of the record. The body weight of this animal showed a more striking reduction from that of the male than that of either rat 2 or 3. At the age of 149 days the animal weighed 210 grams which was about 100 grams less than that of the average male at this age and only a few grams above the average weight of females.

Seven of the twenty-four castrated animals failed to show an increase in activity following transplantation of ovarian tissue. These animals will be referred to as "functional non-takes." A record of one of these animals, rat 12, is presented in figure 4. This animal was put in the running cage at 30 days and was castrated at 70 days. It is seen that up to the time of castration the activity had reached a fairly high level and that after castration there was an immediate marked drop. Ovaries were transplanted when the animal was 80 days old, following which there was neither a change in activity nor in body weight. The average daily activity forty days after the transplantation showed a decrease rather than an increase. The body weight continued its normal rate of increase and at the age of 140 days the animal weighed 350 grams which is well above the average weight even of males at this age.

We found that the younger the animal the better the chances were for successful transplantation. Our unsuccessful attempts may be explained

at least in part by the fact that our animals were already well beyond puberty at the time of transplantation.

The results of our experiments are summarized in table 1 in which detailed records of the seventeen "functional takes" and of the seven "functional non-takes" are given. The identification number of each rat is given in the first column; the average number of revolutions per day for a

TABLE 1

RAT NUMBER	DAILY RUNNING AVERAGE OF CASTRATED ANIMALS FOR TWENTY DAYS BEFORE TRANSPLANTATION	DAILY RUNNING AVERAGE FOR TWENTY DAYS ON LEVEL OF PLATEAU OF ACTIVITY AFTER TRANSPLANTATION	RATIO OF RUNNING AVERAGES OF TRANSPLANTATION TO PRE-TRANSPLANTATION PERIOD	HIGHEST PEAK		DAILY RUNNING AVERAGE FOR TWENTY DAYS AFTER GRAFTS WERE REMOVED	DECREASE IN ACTIVITY IN PER CENT AFTER REMOVAL OF GRAFTS
				Before transplantation	After transplantation		
Functional takes							
9	410	4660* 10510†	11.3 25.6				
2	907	9645	10.6	1400	13000	415	95.7
3	460	8690	18.8	1000	16210	515	94.1
8	660	3095	4.6	940	7500		
Y14		6537			8650	430	93.5
W 1		10955			13800	910	91.4
Y13		4785			8000	380	92.1
14	340	3800	11.2	1100	4100		
6		1425	2.05	1100	2100		
17	70	4280* 8122†	61.0 116.0	100	9500		
16	306	780	2.5	800	1700		
4	370	855	2.3	800	1900	190	77.8
15	580	1390	2.4	1000	2000	190	77.8
7	150	470	3.1	500	1500		
10	560	580	1.03	1000	1200		
19	100	270	2.7	200	1600		
18	766	1003	1.3	1200	2300		
Functional non-takes							
11	750	130	0.17	1000	1200		
12	655	260	0.39	1400	1400		
13	521	250	0.48	1400	1400		
Y11	380	200	0.52	1000	700		
Y12	665	485	0.73	1700	1500		
20	130	100	0.76	200	200		
5	320	100	0.31	700	100		

* First plateau.

† Second plateau.

twenty day period during the pre-transplantation period in the second column; the average level for twenty days of the plateau of activity following transplantation in the third column; the increase in activity following transplantation in percentages in the fourth column; the highest peak of activity reached before and after transplantation is shown in the fifth and sixth columns. In six of the animals the grafts were removed before the end of the experiment, so that in these animals a record of activity was

TABLE 2

RAT NUMBER	CYCLE
	Functional takes
9	3, 3, 3, 4, 3, 4, 3, 4, 2, 2, 2, 2, 2, 3, 4, 2, 6, 3, 3, 4, 3, 4
2	5, 4, 5, 4, 3, 4
3	6, 3, 3, 2, 2, 4, 3, 3, 6, 7
8	4, 4, 3, 2, 3, 5, 6, 3, 2, 5, 5, 3, 4, 4, 9, 3, 4, 4, 3, 2, 4, 6, 5, 3
Y14	5, 3, 5, 3, 3
W 1	6, 2, 2, 4, 7, 4, 3, 3, 4, 5, 3, 5, 2
Y13	3, 5, 2, 3, 4, 5, 3
14	3
6	No cycles
17	5, 4, 2, 2, 3, 4, 3, 3, 3, 3
16	9, 10, 3, 6
4	6, 4, 3, 5, 3, 2, 5, 5, 2, 6, 4
15	6, 3, 13, 8
7	No cycles
10	No cycles
19	No cycles
18	3, 3, 3, 3, 4, 3, 5
	Functional non-takes
11	No cycles
12	No cycles
13	No cycles
Y11	No cycles
Y12	No cycles
20	No cycles
5	No cycles

obtained for a considerable period during which the animal was again in the neutral castrate condition. The average level of activity following the removal of the grafts is shown in the next to the last column. The decrease in the activity is shown in the last column, expressed in percentages.

It will be seen that the increase in activity following transplantation shows a graded effect varying from 1.03 to 116 times the pre-transplantation level. The lowest average was 270 revolutions per day after trans-

plantation, while the highest was 10955. The effect of transplantation is further demonstrated by a marked increase in the height of the peaks of activity after the operation. That these higher running levels were due to the presence of the grafts is conclusively established by the fact that when the grafts were removed the activity immediately decreased again (about 90 per cent) to its original neutral castrate level.

TABLE 3

(1) RAT NUMBER	(2) ACTIVITY	(3) AVERAGE DAILY RUNNING ACTIVITY AFTER TRANSPLANTATION	(4) AVERAGE LENGTH OF CYCLE IN DAYS	(5) BODY WEIGHT	(6) PERCENTAGE NORMALITY OF GENERAL OVARIAN PICTURE	(7) PERCENTAGE NORMALITY OF GRAAFIAN FOLLICLES	(8) PERCENTAGE NORMALITY OF CORPORA LUTEA
W 1	Take	10955	3.9	Take	60	75	60
9	Take	10510	3.1	Take	50	90	20
2	Take	9645	4.2	Take	60	80	50
3	Take	8690	3.9	Take	40	50	10
17	Take	8122	3.2	Doubtful	40	80	20
Y14	Take	6537	3.8	Take	30	5	90
Y13	Take	4785	3.6	Take	60	5	100
14	Take	3800	3.0	Take	30	20	10
8	Take	3095	4.0	Take	40	80	20
6	Take	1425	No cycles	Take	40	70	20
15	Take	1390	7.2	Doubtful	Grafts not studied		
18	Take	1003	3.4	Non-take	Grafts not studied		
4	Take	855	4.1	Take	20	15	0
16	Take	780	7.0	Doubtful	40	100	5
10	Take	580	No cycles	Doubtful	50	80	60
7	Take	470	No cycles	Doubtful	50	75	50
19	Take	270	No cycles	Non-take	10	5	60
12	Non-take	260	No cycles	Non-take	0 (Resorbed)		
13	Non-take	250	No cycles	Non-take	0 (Grafts resorbed)		
Y11	Non-take	200	No cycles	Non-take	Grafts not studied		
Y12	Non-take	485	No cycles	Non-take	Grafts not studied		
11	Non-take	130	No cycles	Non-take	0 (Grafts resorbed)		
20	Non-take	100	No cycles	Take	50	100	10
5	Non-take	100	No cycles	Take	10	10	0

In these experiments it is shown then that the activity of castrated males may be markedly increased by the presence of the ovaries. To what extent does the increase in activity in these castrated males have the characteristics of female activity? Five of the seventeen "functional takes" reached a daily running level of 8000 or more, which is close to the average of normal females, and considerably higher than the average for normal males except in a few instances. We may thus regard these results as fairly conclusive evidence of feminization. In four other animals the new level of trans-

plantation varied between 3000 and 7000 revolutions which is within the limits of variation of the normal male. The eight remaining animals ran considerably less than either normal males or females.

Transplantation of ovaries has a graded effect also on the activity cycles. The records of the cycles of individual animals is given in table 2. Four of the "functional takes" exhibited fairly regular cycles which resembled those of the normal female. Nine of the "functional takes" showed cycles which were very much less regular. There were no cycles in the remaining four "functional takes" nor in any of the seven "functional non-takes."

Twelve of the twenty-four animals showed a definite change in body weight after transplantation of ovaries. The effect of transplantation on body weight shows itself in two ways: first, the rate of growth of the castrated animals, which is normally very much more rapid than that of females, was decreased until the body weight curve closely approximated the normal curve for the female. This is shown most clearly in the marked flattening in the body weight curve of rat 9 in figure 2c. In the second place the effect of the ovaries showed itself in the marked increase which took place following the removal of the grafts. These twelve animals in which the body weight was very obviously altered in this way will he referred to as "body weight takes" in contrast to seven other animals whose body weight remained close to that of the average male throughout the course of the experiment. The remaining five animals showed body weight changes which were somewhat doubtful. These will be referred to as "doubtful body weight takes."

The relation of the "functional takes" to the "body weight takes" is presented in table 3. Ten of the seventeen "functional takes" are "body weight takes." Of the remaining seven "functional takes" five are "doubtful body weight takes" and two are "body weight non-takes." Out of the seven "functional non-takes" five are "body weight non-takes" and two are "takes." In most cases the change in body weight was in direct proportion to the increase in activity. The animals which ran most following the transplantation of ovaries also exhibited the greatest flattening of the body weight curve and the animals which showed the smallest change in activity showed the smallest changes in body weight.

This close relationship between the changes in body weight and activity is demonstrated very definitely in the animals on which records were taken following removal of the ovarian grafts. It has already been pointed out that removal of the grafts causes a large decrease (about 90 per cent) in activity, and an increase in body weight. It is interesting to note that the amount of increase in body weight following removal of the grafts is directly proportional in all instances to the average daily running activity before this operation. This is shown in the following table:

RAT NUMBER	AVERAGE RUNNING ACTIVITY BEFORE REMOVAL OF GRAFTS	INCREASE IN BODY WEIGHT IN PER CENT FOLLOWING REMOVAL OF GRAFTS
		per cent
2	9645	41
3	8690	32
Y14	6537	24
Y13	4785	20
4	855	16

in which the number of the rat is given in the first column, the average daily running level in the second column, and the increase in body weight in per cent thirty days after the operation (the quotient of the amount of increase in thirty days over the body weight at the time the grafts were removed). Rat 2 which ran most before the operation shows the largest gain in body weight, while rat 4 which ran least shows the smallest body weight gain. This finding confirms numerous observations that we have made on the effect on body weight of castration and spaying. The amount of the increase in body weight which follows is proportional to the amount of activity before the operation. It may be pointed out, as an aside, that the failure to take the activity factor into consideration has led to erroneous results, as in the case of the work of Stotsenburg (1909), (1913), (1917) on the change in body weight following castration and spaying. Stotsenburg found that females showed a definite increase in body weight while males failed to show any change at all. We have found that rats which have been active, males as well as females, show an increase in body weight following castration. Males that are almost completely inactive do not show a change. Females are more active than males and for this reason always show a larger increase in body weight following spaying.

The results of the transplantation of ovaries on body weight offer a two-fold interpretation. We may regard the retardation in growth of the castrated male as the direct effect of the ovaries; and we may also regard it as being due to the indirect effect of increased activity produced by the ovaries.

The behavior part of these experiments was limited to the study of the changes in spontaneous activity which occur following transplantation. No attempt was made to correlate these changes with specific male and female sex activity. This is being done in another series of experiments.

To determine if a correlation exists between the behavior of the animal and the condition of the grafted ovaries, one of us (A. F. G.) made a histological study of the transplants. The tissue about the site of the graft was studied in twenty of the twenty-four animals which were used in these behavior experiments. It was thus possible to compare the morphology

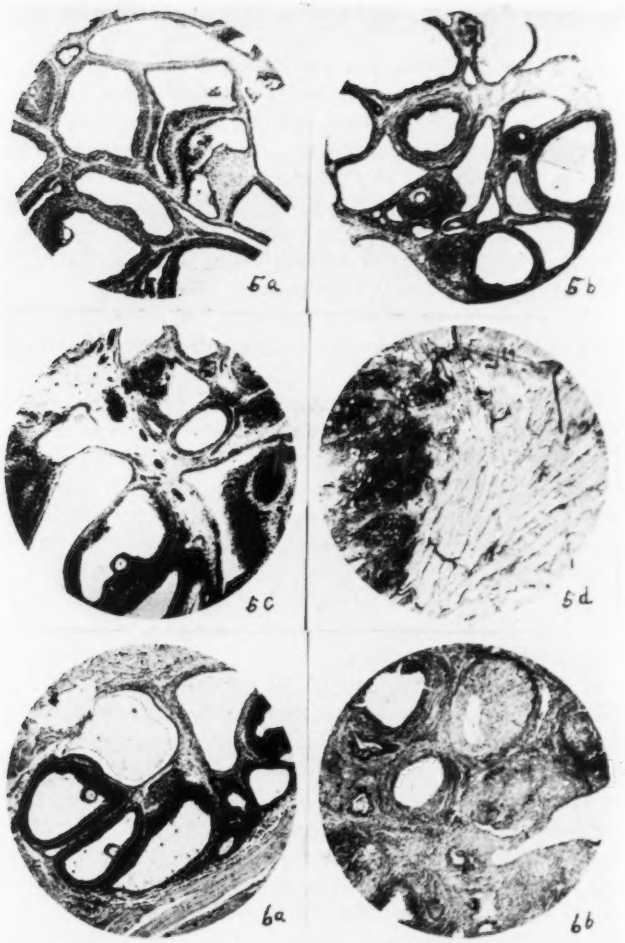
of the grafts in the functional and body weight take, doubtful take and non-take.

At varying intervals following transplantation the rats were anesthetized. In some of the animals the grafted areas were excised and fixed in formalin while in others a vascular injection of india ink was made and the tissue fixed. The graft and the muscle surrounding it were dehydrated, embedded in either paraffin or celloidin, and sectioned at 15 to 30 micra. Most of the sections were stained in hematoxylin and eosin, though many were stained with iron hematoxylin, Mallory and Wright, and carmine.

Unmistakable ovarian tissue is to be seen bilaterally in seventeen of the twenty transplanted animals examined. The grafts of rats 2, 3, 9 are shown in figure 5, a, b and c. In the other three rats (nos. 11, 12 and 13) no vestige of ovary remains on either side; the site of the transplant being only demarcated by the presence of the well healed scar, and the black silk which was used in suturing the graft in place (fig. 5d). In the other seventeen rats ovarian tissue is found bilaterally, well embedded in the recti muscles. In these seventeen pairs of ovarian grafts we find all degrees and types of variation from the histology of the normal rat ovary. In no instance does the transplanted ovary resemble closely the typical normal picture of the ovary of an adult female: little stroma, with multiple corpora lutea in various stages of development and regression, separated here and there by maturing and mature graafian follicles.

After comparing the graft with the normal rat ovary we have attempted to express the degree of normality of the transplant in terms of percentage. We realize that such an empirical procedure is open to objection; but we have fortified ourselves against the less accurate judgment of one observer by asking another worker (G. W. Corner) to evaluate the grafts independently. Table 3 gives the averages of two sets of values made by Doctor Corner and one of us (A. F. G.) along with a summary of the activity and body weight changes for each rat. The activity and body weight records are given in the first five columns. Since we do not know what part of the ovary is responsible for this female activity-phenomenon we thought best to evaluate the general ovarian picture (the size of the graft, the amount of scarring, number of large follicles and amount of lutein tissue) separately from the normality of the graafian follicles and corpora lutea. This we have done in column six, the normal ovary being 100 per cent. In columns seven and eight we have expressed separately the normality of the graafian follicles and corpora lutea.

The introduction of the important variable, the length of time which the graft was allowed to remain in place, makes a very exact comparison of the histological data too uncertain. Some of the grafts were removed twenty days after their implantation while others were allowed to remain over four months. We do not know how the age of the graft affects the indi-



All the photographs are from preparations stained with hematoxylin and eosin, and magnified sixty diameters. Photographs made by Bausch and Lomb.

Fig 5a. Transplanted ovary of rat 2. One of the most normal of the transplants, containing a small amount of lutein tissue and a large number of follicles.

Fig. 5b. Transplanted ovary of rat 3.

Fig. 5c. Transplanted ovary of rat 9.

Fig. 5d. Rectus muscle of rat 12, at site where ovary was engrafted. All ovarian tissue has been resorbed, leaving only scar tissue. Blood vessels injected with india ink.

Fig. 6a. Transplanted ovary of rat 10, which was functionally inactive. Note that the transplant is anatomically more nearly normal than that of rat Y 14, shown in figure 6b.

Fig. 6b. Transplanted ovary of rat Y 14, which was functionally active. Graft heavily infiltrated with scar tissue and mononuclear cells. It appears anatomically less normal than the graft in rat 10.

vidual microscopic picture. It is unfortunate that it was impossible to sacrifice all the animals when the transplants were of equal age.

However there are a few general facts which an anatomic study of the grafts demonstrates. It is significant that the only three rats studied anatomically which did not at any time show either an ovarian effect on their activity or body weight (that is, they were neither activity nor body weight takes), had no ovarian tissue persisting. In these three animals which were totally castrate as far as their behavior is concerned, there was a complete resorption of the ovarian grafts.

All of the remaining seventeen animals studied microscopically showed ovarian tissue present and, according to behavior criteria they were all either activity or body weight females.

Neither the amount of activity shown, nor the average duration of the cycle, which in normal females is 4 to 5 days, seems to bear any definite relation to the degree of normality of the ovarian transplant. Rats 10, 17 and 20 show very slight or no increase in activity following transplantation, have doubtful body weight takes, and no observable cycle; but a microscopic study of their ovaries rates the transplanted gonads as about 50 per cent normal (fig. 6a). On the other hand rats Y 14 and 14 show a much greater increase in activity following transplantation, are definite body weight takes, have oestrous cycles and yet their ovaries are only 30 per cent normal (fig. 6b).

It seems then that the presence of the ovarian transplant feminizes the castrated male in either its activity or body weight or both; that the resorption of the graft makes the animal a neuter castrate. However there appears to be no consistent correlation between the degree of feminization, the extent of activity, or the degree of specificity of the weight curve and the microscopic normality of the transplanted ovary.

Since no gradation in difference could be demonstrated between the general ovarian histology of the strongly and doubtfully functional grafts, it was decided to make a vascular injection of a small number of the male castrated rats in which ovaries had been transplanted. Here again our results are befogged by the variable length of time the grafts were allowed to remain in before the rat was killed. After anesthetization a 1 to 1 dilution of india ink was injected at 80 mm. of mercury pressure through a cannula tied into the left ventricle. The ink was allowed to flow until it ran out of a hole cut in the right auricle. The vascular system was clamped and tied off, and when the rat was dead the whole animal was put in formalin. The site of grafting was excised, dehydrated, embedded, sectioned and stained. Six rats were thus injected, nos. 9, 17, 8, 7, 19 and 20. The richness of the ovarian vascular pattern varied markedly in the six animals but there was no discoverable relation to the degree of feminization (fig. 7).

In these inconclusive experiments it seems that neither the degree of normality of the ovary nor the richness of the ovarian vascular pattern bear a consistent relation to the extent of the behavior feminization of a male castrate in which ovaries have been transplanted. For a male castrate to

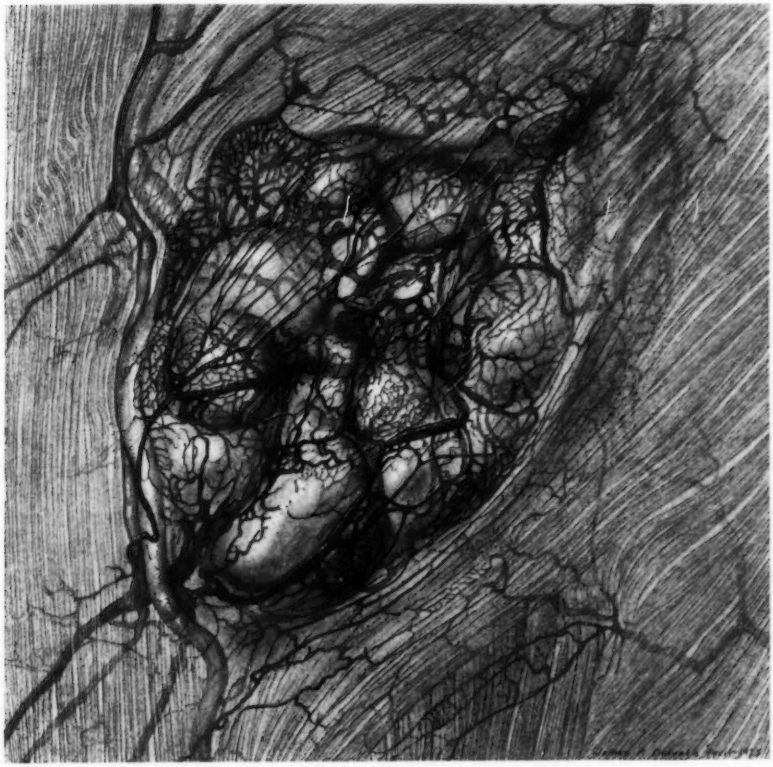


Fig. 7. Cleared india ink vascular injection of graft of rat 9 before microscopic sectioning. Both ovarian vascular pattern and follicles of ovary are evident. Silk suture, which was on under side of specimen, is not indicated in the drawing. Microscopic section of this graft is shown in figure 5c. Drawing made by Mr. James Didusch.

have its behavior transformed into the type characteristic of the female, transplanted ovarian tissue must persist. However, on the other hand, the persistence of ovarian tissue does not feminize the behavior of all of the male castrates. The actual factor upon which successful behavior takes depends, remains to be discovered.

SUMMARY

The basis for these experiments was the observation that the running activity of males castrated at an early age always remains on a low level, and that there are sex differences in activity which can be measured: the four-day cycle present in the female and absent in the male, and the higher running level of the female.

1. Transplantation of ovaries into castrated males may produce a marked increase in activity. The amount of the increase in seventeen out of twenty-four animals varied from 1.03 to 116 times the pre-transplantation level. The remaining seven animals showed a decrease in activity after the operation.

2. Five of the castrated males after ovaries had been transplanted into them ran as much as normal females and considerably more than the average male. Four others reached the average for males, while the rest ran less than either males or females.

3. In four of the animals the activity cycles characteristic of the mature female rat were produced, but were not quite so regular as they are in normal females. Very much less regular activity cycles were present in nine other animals. The remaining animals showed no cycles at all.

4. Twelve of the animals showed a change in body weight following transplantation, that is, the more rapid rate of growth of the castrated males was diminished until the body weight curve closely approximated the body weight curve of the normal female. When the grafts were removed the body weight increased again to the male level. Five animals showed small and somewhat doubtful changes in body weight while seven showed no changes at all in body weight.

5. Histological examination of the grafts showed that ovarian tissue was present in all animals with either a change in activity or body weight. The grafts contained graafian follicles, ova, and corpora lutea, but they did not present the histological picture of completely normal ovaries. The grades of activity and body weight changes were not correlated with definite histological differences in the grafts, except that in animals which showed neither an increase in activity nor a body weight change the grafts were completely absorbed. Because of the absence of any correlation between the activity changes and the histology of the grafts an attempt was made to determine whether the weight and activity changes might not be correlated with the histological picture on the basis of different degrees of vascularization of the grafts. However, no such correlation could be established.

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THE ELECTRICAL RESPONSE TO ILLUMINATION OF THE EYE IN INTACT ANIMALS, INCLUDING THE HUMAN SUBJECT; AND IN DECEREBRATE PREPARATIONS

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The electrical response of the eye, upon illumination, is too well known to require description. It is demonstrated by connecting two nonpolarizable electrodes, placed one upon the cornea of the eye and the other upon its fundus or optic nerve, with a suitable galvanometer. Figure 1, *1*, is a photograph of a typical action current of the excised eye of the frog. Among the numerous papers on the subject, those of Einthoven and Jolly (1908), and of Chaffee, Bovie and Hampson (1923) may be referred to as containing references to the literature, as well as extensive description and figures of the response. Electrical currents set up in the eye-ball in response to both the onset and cessation of illumination have been shown by Dewar and McKendrick (1874) to be the action currents of the retina.

Most of the work heretofore has been done on the excised eye, although the deleterious effects of removing the retina from its blood supply, particularly in mammals, have been recognized. Operations have been devised for exposing the fundus of the eye-ball without removing the eye from its orbit (cf. Piper, 1905, and Day, 1915). It is the object of this paper to report some experiments performed with a view toward obtaining a preparation in which the action currents of the eye may be obtained easily without interference with the retinal circulation.

The source of light is a 400 W. Mazda projection lamp (flat filament type), burning 3.00 amperes from a 120-volt storage battery. An image of the filament, formed by a system of lenses, is focused upon a ground glass screen. Between it and the filament are interposed a rapidly moving shutter and a water cell to remove heat rays. Wratten Neutral Tint Optical filters are used to reduce the intensity of the image; they are put next to the ground glass screen, on the side toward the lamp. The image on the screen is 15 millimeters square, and has a brightness of 43.2 candle power without filters.

The preparation is placed in a light-proof chamber, with the cornea of its eye 2 mm. behind an artificial pupil of suitable size, and 86 mm. from the ground glass screen.

The non-polarizable electrodes are calomel half-cells, made up in saturated potassium chloride, equipped with replaceable tips of kaolin paste and cotton. Connections are made from these electrodes with the thread of an Einthoven galvanometer. In series with the galvanometer is a Williams box for compensating the current of rest. A potentiometer, also connected in series, serves to impress a standard E. M. F. of 0.343 millivolt upon the circuit. The galvanometer thread is a platinum wire, about 85 mm. long, 2.5 micra thick, and has a resistance of 1520 ohms. All leads and connections are encased in grounded metal sheaths, to shield the circuit from stray electric fields.

The shadow of the thread is photographed, in the usual manner, on a moving strip of sensitized paper, together with a time record and the shadow of an electromagnetic signal connected with the shutter. The sensitivity of the thread, and the magnification of the projection apparatus are such that a current of 1.6×10^{-8} amperes gives a deflection of 10 millimeters to the thread shadow. The connections are so made that a current flowing in the external circuit from the electrode placed on the cornea to the other electrode produces a deflection of the string shadow upwards in the photographs. Such a current is termed a positive current, after Waller (1900), since it traverses the eye from fundus to cornea.

EXPERIMENTAL. Action currents have been obtained from the eye *in situ* by opening the orbit, thus giving access to the fundus of the eye-ball but, as far as I know, no one has made use of decerebrate preparations. In such preparations the cut end of the optic nerve is available, inside the cranium, for an electrical connection, and there has been no trauma in the orbit or traction of the eye-ball. The preparations, moreover, exhibit few spontaneous movements, and therefore need be given no drugs. For these reasons, the use of acute preparations of decerebrate frogs and mammals will be described.

Large Louisiana frogs (*Rana catesbiana*) may be operated upon under ether anesthesia; the brain substance anterior to the cerebellum is gently scraped away with a fine glass hook, avoiding injury to the main blood vessels on the floor of the cranium. The retinal blood supply comes from vessels outside the skull. Electrodes are placed one upon the cut end of the optic nerve, inside the skull, the other upon the cornea of the eye. The eye is made to protrude by placing a wad of cotton under it, in the mouth. Action currents obtained from the eye of this preparation persist unchanged as long as the animal remains in good condition, which is usually about a week.

The Sherrington decerebrate cat is an admirable preparation for this work. Nearly all spontaneous movements are eliminated, and the animal lasts for a number of hours. The ophthalmic artery, which supplies the retina, arises from the internal maxillary, but even its anastomotic con-

nections may be left undisturbed by modifying the method of decerebration in the manner described by Bazett and Penfield (1922), a modification made to prevent injury to the vessels supplying the hypophysis. The operation is done under ether anesthesia; a hole is trephined in the skull, and the dura is slit. The carotid and vertebral arteries are temporarily occluded, and the decerebration is performed at the level of the *tentorium cerebelli*, using a blunt spatula. The brain stem is cut through only about two-thirds of the way; the spatula is then turned and brought forward parallel to the floor of the skull. On removing the brain substance, a layer of nervous tissue is left, covering the uninjured arteries and dural sinuses on the cranial floor. This tissue may be carefully pushed back, starting anteriorly, and the optic chiasma exposed, isolated from the optic tracts and cut across. If this last procedure is carried out in several stages after the decerebration, the bleeding in the cranium will stop and the dissection may be done without injury to any vessels.

The eyelids are held open with adhesive plaster tape, and action currents obtained from the eye, leading off from cornea and cut end of optic nerve. Figure 2, *1*, is a typical response.

Prepared in such a manner, and fourteen hours after decerebration of the animal, I have obtained action currents from the eye of a cat which are similar in character and magnitude to those obtained immediately after recovery from anesthesia. This is evidence that there has been no injury to the retina, inasmuch as the action current deteriorates very rapidly after death. This deterioration may be easily shown by stabbing the heart, and, after death supervenes, observing the action current at intervals. The responses are markedly changed, both in magnitude and character, three minutes after death; ten minutes after death they are extremely feeble and slow. They resemble the "abnormal response curves" figured by Chaffee, Bovie and Hampson (1923). The excised eye, with better opportunity for gaseous diffusion, probably does not die so rapidly.

There is, in all probability, no more danger of injury to the retina by the method of decerebration than by the method of exposing the fundus of the eye through an operation on the orbit.

In the course of these experiments, I have found that the electrode on the optic nerve did not necessarily have to be confined to that point, but that one could, in fact, place it anywhere on the animal's body and still obtain electrical responses to illumination. I have obtained them (from both mammals and frogs) with this "indifferent" electrode placed on the cornea of the other eye, on the intact skin of the head or fore-paw, in the mouth, and on the cut surface of the head muscles. This suggested the use of intact animals which, it was found, could be used just as well. The qualitative resemblances of the responses so obtained to the action

currents obtained from the eyes of decerebrate preparations or from excised eyes left little doubt but that they were the action currents of the retina. I have since found that Dewar (1877) has described electrical responses to illumination obtained from the intact animal by this method. He states that they are similar to the responses obtained from excised eyes. How such a method, which is obviously valuable in that it supplies a means of studying the action current of the retina without the slightest danger to its blood supply, and with the minimum of experimental procedure, could have remained unused for so long a time, I cannot imagine. And yet the literature consists practically entirely of studies in which the eye, if not excised, has had its fundus exposed by a time-consuming operation, endangering the circulation and ruining the animal for future use. The application to the human subject, which was also made by Dewar (1877) has remained uninvestigated, so far as I know.

The possibility exists, of course, that the electrical responses obtained from the intact animal have an origin other than the retina; for example, they may be the result of reflex movements of the eye-ball, or eye-lids. The proof that they are the action currents of the retina lies in a consideration of the preparations used and particularly in a qualitative and quantitative comparison of the results obtained by different methods. Dewar, using a slow-moving galvanometer, could not present such proof as will now be given.

Large Louisiana frogs are naturally lethargic; they may easily be tied down, and remain quiet without the use of drugs. In fact, they are superior to the decerebrate frog, as nearly all spontaneous movements seem to be inhibited. They do not react, by blinking the eye, either to the electrode on the cornea or to the sudden flash of the brightest light. The small frogs (*Rana pipiens*) are best used after being curarized. At the risk of affecting the retina Dewar used chinoline to quiet the frogs with which he worked. He did, however, curarize the fish he studied.

Figure 1, *2*, is a photograph of the electrical response to illumination of the eye of a frog, obtained by placing an electrode on the cornea of each eye. This animal was intact, save for a small piece of skin removed from the back of the orbit of the illuminated eye for the purpose of exposing the fundus. Immediately after this photograph was taken, the electrode on the dark eye was moved so as to rest upon the fundus of the illuminated eye. The response obtained upon illumination is not figured; it is in all respects practically identical with figure 1, *2*. Figure 1, *1*, is a photograph taken a little later, of the response from the same eye after removing it from the frog. The conditions of adaptation and stimulation were the same in these cases, and the qualitative resemblance,—direction of waves, their number, relative magnitudes, and time relations, is apparent. Figure 1, *3*, is a response obtained from an intact frog, immobilized by curare.

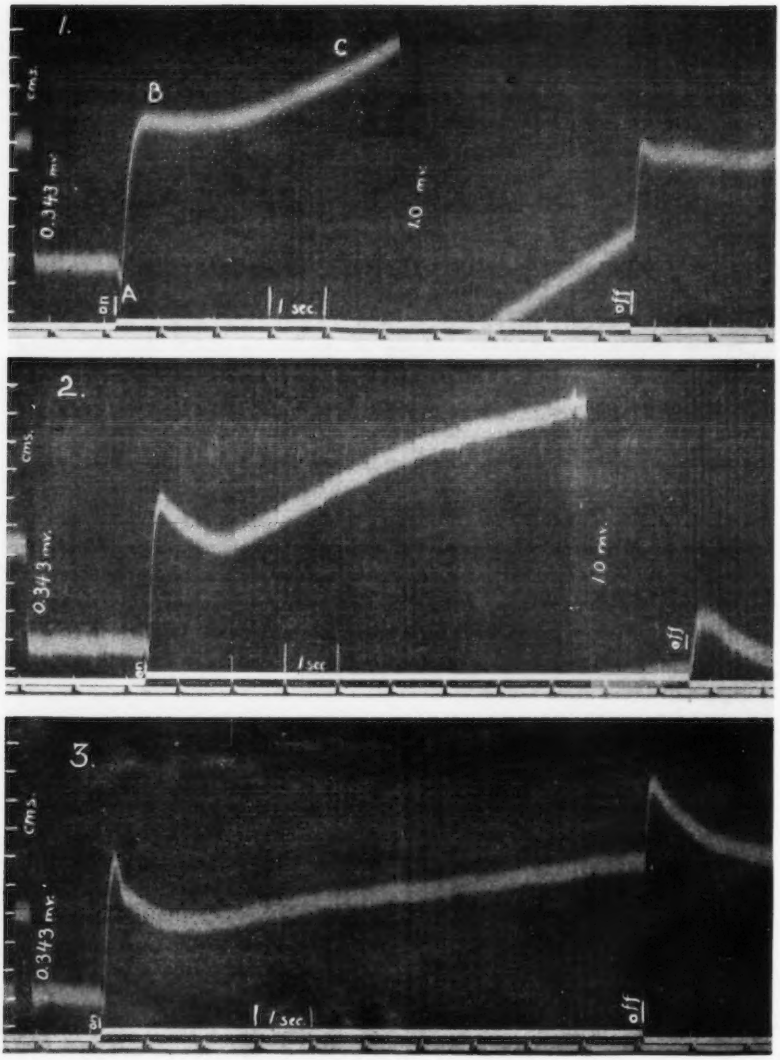

Fig. 1. Action currents of the frog eye, obtained by different methods. Lower line: time record, in seconds. Middle line: signal marking instant of stimulation—movement upward denotes onset, downward, cessation of illumination. Upper line: shadow of string. Deflection upwards denotes an increase in the electrical positivity of the cornea of the eye under investigation. 10 mm. = 1.6×10^{-8} amps. Intensities of stimulating light: I = 1.0 is a brightness of 43.2 candle power. Distance from source to cornea of eye: 83 mm. Area of source: 225 mm.^{2} Area of artificial pupil: 4.5 mm.^{2}

1. Frog 12. Excised eye. Leads: cornea—fundus. I = 0.1 Temp.: 23°C.

2. Frog 12. Intact animal. Leads: eye—eye. I = 0.1 23°C.

3. Frog 9. Intact animal, curarized. Leads: eye—eye. I = 1.0 20°C.

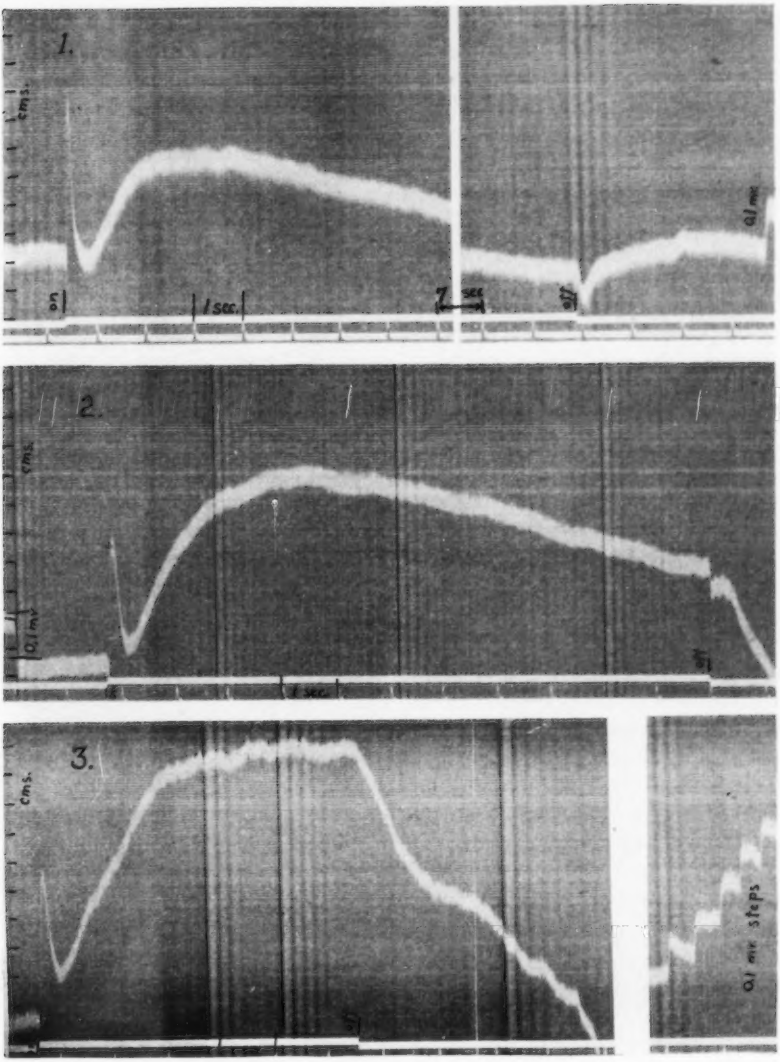

Fig. 2. Action currents of mammalian eye, obtained by different methods. Mydriatic: atropin. Area of artificial pupil: 11.2 mm.^{2} Otherwise as in figure 1.

1. Cat. Decerebrate animal. Leads: cornea of eye—cut end of optic nerve, inside the skull. I = 0.08 Temp.: 44°C. (rectal temp.).

2. Rabbit 3. Decerebrate animal. Leads: cornea—optic nerve, as in *1*. I = 0.08 38°C (rectal temp.).

3. Rabbit 3. Intact animal. Leads: eye—eye. I = 1.0 38°C.

In figure 2, *2* and *3*, is a similar comparison of the response obtained from an intact rabbit, leading off the electrical currents from the corneas of the two eyes, with that obtained from the same rabbit after decerebration, leading off from the cornea and optic nerve of the illuminated eye. In this case the conditions of adaptation are the same for each curve, but the intensity of stimulus is different. Nevertheless, a qualitative comparison can still be made. The rabbit is the only intact mammal which I have been able to tie down and use successfully, undrugged. It is a difficult preparation to handle, as the electrode irritates the cornea and the brighter lights causes reflex movements of the eye and lids. By using a little care, however, quite good results may be obtained.

I have also obtained a typically "abnormal" response from the eye of an intact cat a few minutes after death.

The human subject was used with success by Dewar (1877). I have devised two methods of leading off the electrical responses to illumination of the eye of the human subject. By the first method, fluid confined in water-tight compartments bathes the surface of each eye. A pair of goggles is equipped with paraffin rims, molded to fit the face about the eye. A water-tight seal is made with vaseline, and each compartment filled with sterile 0.9 saline. A tube leading to the bottom of the compartment permits a fluid junction between the saline bathing the eye and the potassium chloride solution of the electrode. The conjunctivae are thus connected electrically. The subject lies on his back, his head resting on a rigid support. By means of a prism, light from the source is reflected into the eye, through the solution bathing it. Image formation is made possible by substituting convex lenses for the plane glasses of the goggles. This is important, in order to make possible fixation (upon a small red light) during the period of darkness. Lid movements produce troublesome electrical changes, and as they frequently occur reflexly upon illumination of the eye, they tend to mask the true electrical responses. This method has the further disadvantage that the "different" electrode, on the illuminated eye, is not confined to the cornea. The responses obtained are not very typical of action currents of the eye, but are in the right direction. One is figured in figure 3, *7*.

The second method of obtaining electrical responses from the human eye is by anesthetizing the conjunctiva with holocaine. This drug is used clinically, has no harmful effects, does not constrict the pupil and does not affect vision. After the application of three to four drops of a 2 per cent solution, instilled into the eye twice during ten minutes, the conjunctiva becomes insensitive to touch, and the cotton tip of the electrode may be laid upon the cornea. The effect lasts about thirty minutes. The other electrode is best placed in the mouth. The situation is thus exactly comparable to that in the intact rabbit.

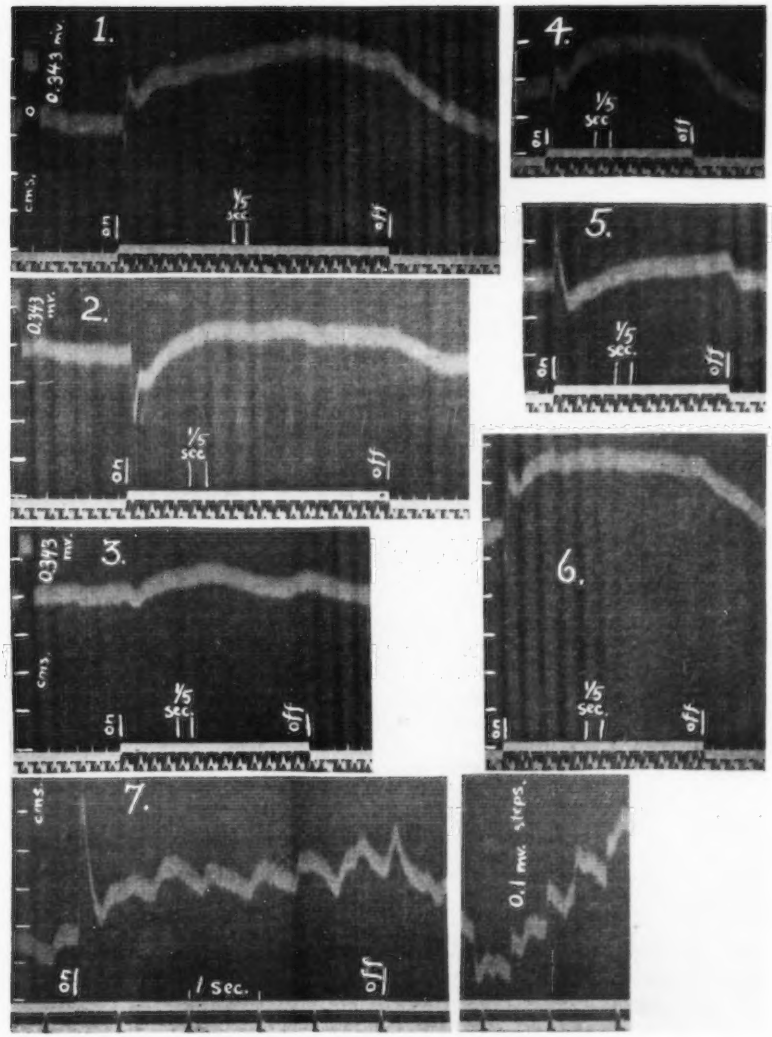

Fig. 3. Action currents of the human eye. Time in fifths of seconds, or seconds, as indicated. Otherwise as in figure 1.

1. Subject. Geschickter. Anesthetized conjunctiva. Leads, cornea of eye—mouth. Distance from source to cornea, 40 cm. No artificial pupil. I = 0 01. Time in fifths of seconds. No dark adaptation.

2. ditto; I = 0.01; *3.* ditto; I = 0.0001; *4.* ditto; I = 0.01; *5.* ditto; I = 1.0; *6.* ditto; I = 0.01.

7. Subject: Gilbert. Method of water-tight goggles; eyes bathed in saline. Leads, eye—eye. Distance from source to cornea, *ca.* 50 cm. Area of artificial pupil, 11.2 mm.^{2} I = 1 0. Time in seconds. Dark adaptation, 20 minutes.

Reflex movements of the eye-ball and eye-lids are more active in the human subject that in the rabbit, but are less troublesome in the second method than in the first. Bright lights cannot be used at all, and even with those of lower intensity it is better if the eye is moderately light adapted, and the subject accustomed to the flashes, since the results are then not so apt to be masked by a sudden wink. Figure 3, *1* to *6* are photographs of the electrical responses to illumination obtained in this way. The same general qualitative features are to be noted—the responses possess all the features of the action current typically obtained from the mammalian eye. The special prominence of the "A" wave is explained by the fact that this wave is more marked in the light-adapted than in the dark-adapted eye (see Einthoven and Jolly (1908), discussion of "The First Substance").

TABLE 1

The electrical response of the frog's eye (A — B waves) obtained by various methods. Intensity (I = 1.0) equals 43.2 C.P. Distance from light source to artificial pupil, 8¼ mm.; from light source to cornea of eye, 86 mm. Area of artificial pupil, 4.5 mm.²

ANIMAL	PREPARATION	LEADS	I	RESPONSE —MV.
(a) Frog 4	Intact	eye–eye	1.0	0.65
	Decerebrate	eye–skin of head	1.0	0.67
	Decerebrate	eye–eye	1.0	0.65
	Decerebrate	eye–eye	1.0	0.61
	Decerebrate	eye–optic nerve	1.0	0.62
(b) Frog 12	Eye in situ, animal intact but for exposure of fundus	eye–eye	0.1	0.53*
		cornea–fundus	0.1	0.47
	Excised eye	cornea–fundus	0.1	0.45**

* Figure 1, *2*.

** Figure 1, *1*.

For a quantitative comparison of the responses obtained by different methods, the magnitude of only one of the deflections will be used. Probably the most constant of these is the sudden electrical effect at the onset of illumination. The combined magnitudes of the "A" and "B" waves, in response to a given illumination, can usually be reproduced in the same preparation within 10 per cent, if conditions of adaptation remain the same. In the following experiments the eye was first exposed to the brightest light for a certain length of time (a few seconds), and then allowed to remain in the dark for ten minutes or preferably twenty minutes, as the case may be.

Table 1, a, gives the magnitudes of the combined A–B waves of the electrical response to illumination, obtained from a large frog by different

methods. The electrical variations between the cornea and any point on the body, either in the intact or decerebrate animal, agree quantitatively with those of the action current of the eye left *in situ*, lead off from the cornea and optic nerve. Table 1, b, gives the magnitude of the responses reproduced in figure 1, *1* and *2*, obtained as described above. The electrical response of the eye, obtained from the intact animal, is in agreement both quantitatively and qualitatively with the action current of the excised eye, or the eye left *in situ*, lead off from cornea and fundus.

A similar comparison, using two widely differing intensities, is made for the rabbit in table 2; before decerebration the electrodes were placed on the corneas of the two eyes, after decerebration they were placed on the cornea and optic nerve of the illuminated eye.

The relation between intensity of illumination and magnitude of electrical response of the retina is known. The diagram, figure 4, gives the

TABLE 2

Electrical response to illumination of the rabbit's eye (A–B waves) obtained by two methods. Intensity (I = 1.0) equals 43.2 C.P. Distance from light source to artificial pupil, 84 mm.; from light source to cornea of eye, 85 mm. Area of artificial pupil, 11.2 mm.² Animal, rabbit 3.

PREPARATION	LEADS	I	RESPONSE—MV.
Intact	eye–eye	1.0	0.30
		1.0	0.30
		1.0	0.39
		0.0057	0.14
Decerebrate	eye–optic nerve	1.0	0.37
		0.0057	0.69

values of the electrical response (A–B waves), of the frog eye, obtained from the intact, undrugged animal, plotted against the logarithm of the stimulating intenstiy. It compares favorably with curves published by Chaffee, Bovie and Hampson (1923), and by Chaffee and Hampson, (1924), relating the first part of the action current of the excised retina to the intensity of illumination.

Discussion. From the foregoing evidence we find that electrical changes occur upon illuminating the eye, that may be recorded from the intact or decerebrate animal by placing one electrode upon the cornea of the eye and the other upon any moist surface of the body. These responses resemble the action currents of the retina, obtained from the eyeball alone, either after excision or, by suitable methods, *in situ*. The resemblance is in direction, in qualitative detail, and quantitatively in magnitude and in time relations. As Dewar states (1877) the responses are not obtained unless the retina is in the circuit. That they are not

the result of extrinsic movements is shown by their persistence in animals immobilized by decerebration, by curare, or by death. It may be concluded that their identity with the action currents of the retina is thus established.

That the action currents should be conducted in such a manner is to be expected. The cornea of the eye is entirely a free surface, while the fundus and optic nerve are closely connected electrically with the rest of the body. The body of the animal is equivalent to a pad of cotton or filter

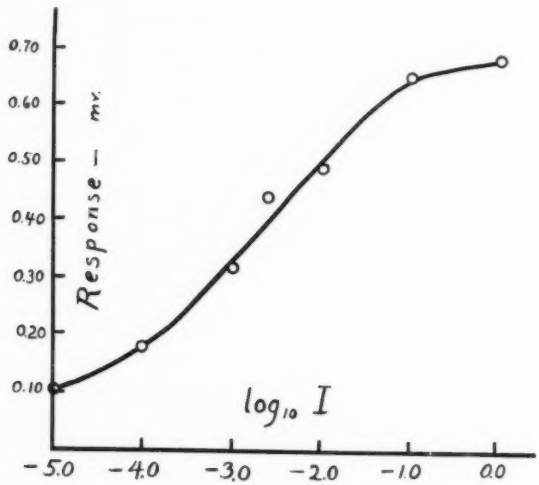

Fig. 4. Magnitude of the electrical response to the onset of illumination of the frog's eye *in situ* in the intact animal, at different intensities of illumination.

Frog 6. Large Louisiana variety. No drugs. Leads: eye—eye. Distance from cornea of eye to source of light: 86 mm. Area of light source: 225 mm.^{2} Area of artificial pupil: 4.5 mm.^{2} Intensities of source: I = 1.0 ($\log_{10}$ I = 0.0) is a brightness of 43.2 candle power; 20 minutes dark adaptation, starting from an exposure to I = 1.0 for 10 seconds, between experiments. Different strengths of stimuli taken in random succession. 21°C.

Abscissae: Logarithms of relative intensities of stimulating lights.

Ordinates: Electrical response (A–B waves) of eye, millivolts.

paper, soaked in saline and applied closely to the fundus and optic nerve of the eye.

It is well to clear up a point which has persisted in the literature in spite of Dewar's paper; it is stated that Dewar and McKendrick (1874) traced the action current to the brain. This they did by placing one electrode upon the cornea of the eye and the other upon the cut surface of the optic lobes. But, as Dewar says in his later paper (1877), the action current of the eye may be traced to any part of the body. In order

to trace the nervous excitation to the brain, another method must be employed.

It is hoped that this study will call attention to the method of obtaining action currents from the eye of the intact animal, and that all doubts as to the nature of the responses so obtained may be dispelled. An animal in which the fundus is exposed by an operation on the orbit must be given some drug such as curare to immobilize it; and since the "indifferent" electrode may be placed anywhere, the animal may just as well be used without the operation on the orbit. An operation may be helpful, however, in immobilizing the animal without the use of drugs, as in the decerebrate preparation. Such an operation is not done to gain access to a location for the "indifferent" electrode but only for the sake of immobilization. Choice between the two preparations will depend largely upon whether the experimenter thinks the intact, curarized animal, or the decerebrate, undrugged preparation most nearly preserves normal conditions in the retina. The fact that curare, especially a poor sample, is without effect on the retina is perhaps not as firmly established as one might wish. On the other hand, the possibility in the decerebrate animal of modifying the circulation in the retina by purely mechanical elements, such as the destruction of intracranial pressure with its effects on the anastomatic connection of the ophthalmic artery, is by no means absent. The general condition of the preparation is in both cases considerably removed from the normal.

With regard to the action current from the human eye, the method, though difficult, may be made to yield quantitative results with a little patience. My photographs, taken even without the precaution of dark adaptation, show a qualitative and quantitative change with intensity of illumination. The value of using the human eye lies in the coöperation of the subject, and in the comparison that can be made between the objective electrical responses and the subjective states. Thus my subject fixated upon a point of red light placed on the edge and halfway from the bottom of the source of light. The source is 15 mm. square, and was 40 cm. from the eye. Its image therefore fell, in all probability, entirely within the rod-free area of the retina, and the action currents represent largely, if not entirely, cone responses. My subject reported a positive after-image, lasting as long as the galvanometer thread remained above the level it finally reached after the cessation of illumination. Now, both the "C" wave and the positive after-image occur even after brief exposure to light, and possess approximately the same time relations. Their possible relation can be established by this method. Other comparisons, such as the extinction rate of intermittent illumination, the course of light and dark adaptation, etc., suggest themselves as possible fruitful fields for research.

I desire to express my gratitude to Dr. C. D. Snyder for his helpful advice and guidance in pursuing this work, as well as for placing facilities for experimentation at my disposal. To Messrs. E. H. Caldwell, J. H. Kupp and Charles Geshickter I am indebted for their services as subjects.

SUMMARY

1. Decerebrate frogs and mammals may be used, in studying the electrical responses to illumination of the eye, as preparations in which the blood supply to the retina has not been disturbed.

2. Action currents from the eye of such preparations are obtained by placing non-polarizable electrodes on the cornea of the eye, and on the cut end of the optic nerve inside the skull.

3. Decerebration serves to immobilize the animal, in mammalian material, and hence no drugs are needed.

4. Electrical responses to illumination may also be obtained, from the intact animal or the decerebrate preparation, by placing one electrode on the cornea of the eye and the other on any moist surface of the body.

5. These responses are in the same direction as the action currents of the excised eye or the eye left *in situ*, and possess the same qualitative details.

6. The responses to a given illumination, obtained in this manner from an intact or decerebrate animal agree quantitatively with the action currents of the eye.

7. It may be concluded, from 5 and 6, that the responses obtained in this manner from the intact animal are identical with the action currents of the eye.

8. The method of obtaining the action current from the eye of an intact animal, in addition to furnishing a preparation in which the blood supply to the retina is unimpaired, also permits the use of the human subject.

9. The human subject is of value because he can coöperate intelligently, and so help the experimenter in many ways, as, for example, in making a comparison between objective retinal responses and subjective states.

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THE NATURE OF CONDUCTION OF AN IMPULSE IN THE RELATIVELY REFRACTORY PERIOD

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In the course of our experiments on the nature of the waves that appear on the falling phase of the potential curve of a mixed nerve during activity, the possibility was considered that these waves might be repetitive processes. This led to a study of the nature of the process travelling in a nerve whose functions were altered by the recent passage of an impulse. While it soon became evident for other reasons that these catacrotic waves are not repetitive processes, the data obtained from this study confirmed this conclusion since the velocity of the β and γ waves is constant (Erlanger, Gasser and Bishop, 1924), while, as will be shown, the conduction of a process in a partially refractory nerve is accelerated.

This subject had previously received several valuable investigations, but as the present data were obtained by different methods, allowing readings accurate to shorter periods of time, as they do not involve the passage of the impulse to muscle, as they give information on mammalian nerve at body temperature, on the nature of the second process under the stimulating cathode, on the mode of return of conductivity, and on the change of form of the second process on conduction, they are collected for analysis in this paper.

If the interval between two stimuli applied to a nerve be short enough, the second is without effect; it neither produces summation in a muscular contraction (Boycott, 1899) nor a second electrical response in nerve (Gotch and Burch, 1899). The nerve during this period is made absolutely refractory by the passage of the impulse (Bramwell and Lucas, 1911).

To express more clearly exactly what is meant by the absolutely refractory period, it is better to adopt the definition of Forbes, Ray and Griffith (1923). They point out that a stimulus has an appreciable duration, also, in view of the fact that subminimal stimuli may be summed, that the stimulus may set up a local process. As a result, the stimulus falling within the refractory period, or the effect of such a stimulus, may last until the nerve recovers, thereby producing a second response. In fact, a

single induction shock may be long enough to produce two disturbances. Therefore, if the absolutely refractory phase is to have any definite meaning, it must be defined not in terms of the stimuli but of properties of the nerve itself, and they propose that the absolute refractory period be defined simply as the time in which the tissue is unable to respond; a definition which fits more exactly with the facts as they are ordinarily conceived.

If the interval between the stimuli is slightly longer, the irritability and conductivity return, the former over a curve which has been determined by Adrian and Lucas (1912). . This second period is known as the relatively refractory period. The latter observers found that the second response is of subnormal intensity, as is shown by its lower ability to pass a decrement. As the interval between the stimuli be increased the ability of the second response to travel through a region of decrement increases, therefore indicating a greater intensity. While the method by which the measurements were made has recently been called into question by Kato (1924), the conclusion in regard to irritability is correct as the increase was shown in capillary electrometer records of nerve by Gotch (1910) and of muscle by Keith Lucas (1911).

It was further shown by Gotch that, if two stimuli be applied to the nerve and a lead be made at a distance to the capillary electrometer, the second of the two processes is delayed, the delay being greater the shorter the interval between the stimuli. This was later shown by Keith Lucas (1911) on a frog sciatic-gastrocnemius preparation, and the fact that it could not be confirmed by Samojloff (1912) was correctly attributed by Lucas to experimental error, as there is no question of its correctness. Gotch concluded that the delay was mainly near the stimulating electrode, since it could not have been in the part of the nerve connected with the electrometer electrodes as the time relations of the second response approximately equalled the first, whereas if the conduction were being delayed at this point, the wave would have been prolonged (his lead being diphasic). He explained the delay by retarded propagation caused by two factors: one physical, which he called "stimulation fatigue," occurring particularly in cooled nerve and being the local result of the stimulus: the other physiological, due to the previous passage of an impulse over the nerve. While Lucas left the explanation of the delay an open question he did not accept that of Gotch, pointing out that slowed conduction would cause the second impulse to lag behind the first, so as to come to follow it at a fixed interval equal to the duration of the modification which the first stimulus had set up. As he had shown, that as the interval between the stimuli is decreased, the delay increases gradually at first, he did not consider Gotch's explanation adequate. He further demonstrated that "stimulation fatigue" plays no part in the production of the delay.

Since the experiments described in this paper were performed, and since the first draft was written, a paper has appeared by Forbes, Ray and Griffith (1923). They found a curve of delay very similar to Gotch's and also demonstrated that the second response is conducted more slowly than the normal, a finding with which the present experiments are in accord. They readily disposed of Lucas' objection by pointing out that whether or not the two processes will appear at a fixed interval depends on where they are observed. If the nerve be examined close enough to the stimulus so that the delay is still taking place, then the delay would increase as the interval of stimulation is decreased. On the other hand, they did not find enough slowing of the conduction of the second response to convince them that it is the only cause of the delay, and therefore postulated, in addition, a delay in the production of the response by the stimulus. With this assumption the experiments here described are not in accord and the question will be discussed later in the paper.

In the present investigation the results are based on observations of the electrical response by means of the Braun tube. The argument is presented largely in the form of an analysis of two experiments: one on the sciatic nerve of the bullfrog, the other on the phrenic nerve of the dog. They may be considered as type experiments, since the results are amply confirmed by data from other nerves. In addition certain points are clarified by specially designed experiments.

CONDUCTION OF A SECOND RESPONSE IN THE PHRENIC NERVE OF THE DOG. The first experiments were done upon the phrenic nerve of the dog on account of its homogeneity and the long fiber obtainable. The fresh nerve from a dog was mounted in a moist chamber according to methods previously described (Gasser and Erlanger, 1922), and the standing waves appearing on the screen of the Braun tube were recorded by tracing them on transparent paper placed over the screen of the tube. Both the first and second stimuli were break induction shocks, rendered very short by high secondary resistances. They were applied rhythmically from a rotating contact-maker in the primary circuit, at intervals of about 30σ. The stimuli were applied through pairs of platinum electrodes placed at intervals along the nerve. Each pair could be connected at will by means of a system of switches to the secondary coils of the two inductoria. The strength of the stimulus was determined empirically for each coil at the beginning of the experiment with the secondary of the other coil in shunt. The coil that furnished the stimulus for the second process was set so that the shock would be super maximal; then the secondary of the coil producing the first process was set so that its shock would be stronger than that of the second, to prevent any chance of the second shock stimulating fibers which had not been previously active. The positions of both secondary coils were then maintained constant throughout the experiment,

as moving either secondary coil would alter the strength of the shock from the other. Care was taken that for each coil the electrode nearest to the proximal lead was cathode for the break shock. Leads were made with non-polarizable electrodes of the Zn-$ZnSO_4$-NaCl type. Their position was in each case constant throughout an experiment, being monophasic, i.e., from the killed end and the side of the nerve. The distance of conduction, as recorded, was always measured from the cathode of the

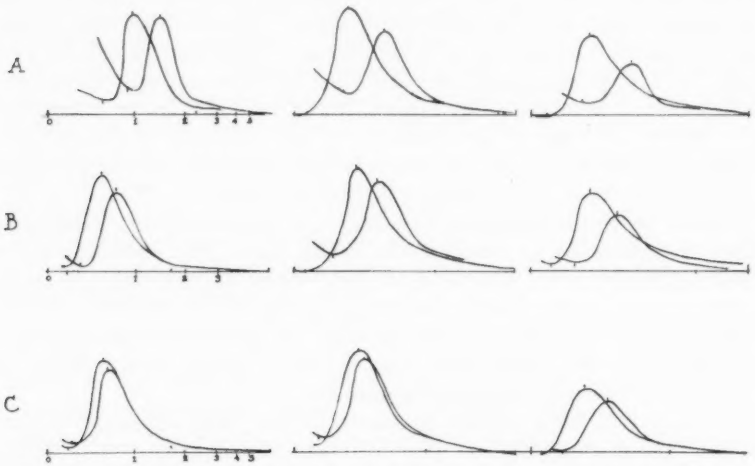


Fig. 1. Action currents from the phrenic nerve of the dog showing delay of an impulse traveling in a refractory nerve. Reproduction ($\times \frac{1}{2}$) of lead pencil tracings made on transparent paper placed directly over the screen of the tube. Stimuli: supermaximal break induction shocks. 2000^{ω}, 1 mf. On some records the time is marked in sigma. The time in the others is the same. The upper row is made with an interval of 0.90σ between the stimuli, the middle with 1.17σ and the lower with 1.62σ. In the first column the distance of conduction is 2.5 cm., in the second 5.9 and in the third 8.73 cm. In the first column the records were made with a $47{,}500^{\omega}$ non-reacting shunt across the nerve. The other records were made without shunt. Normal velocity of the action current about 40 m.p.s.

stimulating electrode to the nearest edge of the proximal lead to the recording apparatus.

The stimuli were controlled by means of a rotating interrupter. The brush controlling the first of the two stimuli was so mounted on a floating arm of the shaft of the apparatus, that its position could be altered with respect to the brush controlling the second stimulus by means of an adjusting screw. The adjusting screw carried a pointer so that its position could be read, thereby allowing a determination of the time interval from a previous calibration. The end of the absolutely refractory period was

determined by trial, as the shortest interval at which the second of the two stimuli produced any action current whatever. The second stimulus was shown by measurement to last about 0.16σ, up to the point at which the potential had nearly returned to zero, a point which certainly represents the termination of any stimulating value. The stimulation interval may therefore be considered as within 0.16σ of the period within which the nerve cannot respond to a second stimulus. The interval between the first and second stimuli was maintained constant, and in rapid succession action currents were recorded at different distances of conduction, three such series being made at the intervals of 0.90, 1.17 and 1.62σ respectively. In figure 1 are found reproductions of tracings from this experiment. The records appear in pairs, the first tracing of the pair giving the position and shape of the action current produced by the "second" stimulus when not preceded by a first action current, the second tracing of the pair giving

TABLE 1

SEPARATION OF STIMULI		DELAY OF THE SECOND PROCESS FOR THE FOLLOWING DISTANCES OF CONDUCTION				TIME TO MAXIMUM FOR THE FOLLOWING DISTANCES OF CONDUCTION		
		2.50 cm.	5.90 cm.	8.73 cm.		2.50 cm.	5.90 cm.	8.73 cm.
σ		σ	σ	σ		σ	σ	σ
0.90	Start	0.37	0.48	0.49	Normal	0.35	0.49	0.51
	Crest	0.46	0.54	0.59	Refractory	0.44	0.54	0.61
1.17	Start	0.19	0.24	0.27	Normal	0.39	0.49	0.48
	Crest	0.19	0.33	0.36	Refractory	0.40	0.58	0.57
1.62	Start	0.04	0.12	0.23	Normal	0.40	0.49	0.52
	Crest	0.06	0.11	0.27	Refractory	0.42	0.50	0.56

the action current produced by the second stimulus when preceded by a first at the designated interval. The records were so made that the start of the action current was always recorded. As has been pointed out by Keith Lucas (1911) and by Samojloff (1912), the delay of the second action current should be measured at the point where there is the first deviation from the action current produced by the first stimulus alone. This point is difficult to read in any case and is more difficult without the first action current for reference, as is possible in a subsequent series; but with long familiarity with such records it is possible to locate the start with some accuracy. On the other hand, since the nerve is composite, with constituents conducting the impulse at slightly different rates (Erlanger, Gasser and Bishop, 1924), the crest of the fused waves changes its position independently of any one constituent. This makes it all the more necessary to use the start of the wave, which represents something more definite: the time of arrival under the electrode of the fastest constituent.

The positions of the starts and crests were carefully measured, and from these data the delays of both the starts and crests, at the different distances of conduction and different positions in the refractory period, were calculated. The durations of the rising phase of the action current were also determined under the different conditions. The results are presented in table 1, and the relationship between the delay of the second impulse and the distance it has been conducted is plotted in figure 2.

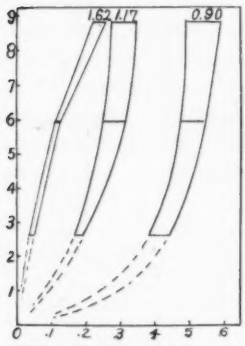


Fig. 2. Graph from the data in table 1. Abscissae in sigma: time lag of the second action current behind its normal conduction, when preceded by a first stimulus at the indicated intervals. The left ends of the lines show the lags of the starts, the right ends the lags of the crests. Ordinates, in cm.: distance the impulse had been conducted when the lag was determined. Solid lines connect the real points. For the basis of the extrapolations (dotted lines) vid. text.

These data indicate that, as conduction proceeds along the nerve, the duration of the rising phase increases both in the case of the normal impulse and of the impulse travelling in a partially refractory nerve, the duration being longer in the refractory nerve at all intervals of separation of the first and second stimuli, and at every distance that the disturbance has been conducted. It must necessarily follow that the crests will be more delayed than the starts, according to the values given in the first part of table 1.

CONDUCTION OF A SECOND RESPONSE IN THE SCIATIC NERVE OF THE BULLFROG. It remains to be seen that these indications were corroborated by subsequent experiments. Before making a detailed analysis of these data the results of a similar experiment made upon the sciatic nerve of the bullfrog, *Rana catesbiana*, will be presented. The method was the same as in the preceding series. At a constant separation of the first and second stimuli, records were made in rapid succession at five distances of conduction with the leads at a constant position. The records were made as contact prints on film placed on the screen of the tube. In this series, in addition to the records of the "second" action current alone, and of the second preceded by a first, records were also made of the first alone. This aids in the determination of the position of the start, an aid which is more necessary in the case of the bullfrog's sciatic on account of the confusion caused by the β wave. At the shortest distance of conduction, the size of the "escape" was so great, that its shape was determined at the close of the experiment, by recording it after the nerve had been killed between the stimulating electrode and the

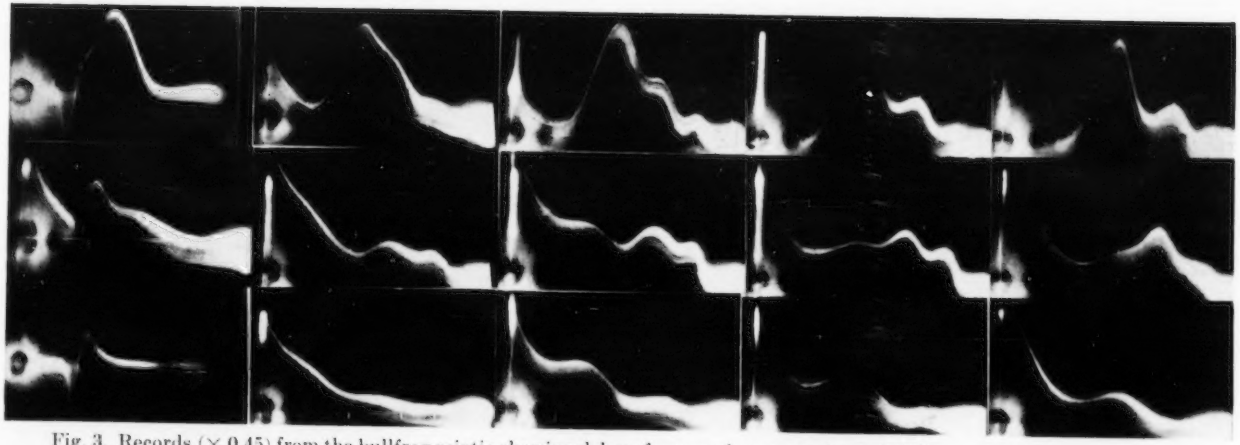


Fig. 3. Records ($\times$ 0.45) from the bullfrog sciatic showing delay of a second process. 1 mf., 2000$^{\omega}$.

Upper row: The "second" action current not preceded by a first.

Middle row: The same action current when its stimulus had been preceded by another 1.39σ previously. Absolutely refractory period ends at 1.2σ.

Lower row: End of the first action current which rendered the nerve refractory, recorded to aid in the determination of the starts of the action currents (the second) in the middle row. Lower record of the first column gives the form of the escape at this distance. The columns from left to right show the action currents at the conducting distances of 1.2, 3.1, 4.65, 6.25 and 8.2 cm. respectively. The records in the first column were made with a 5930$^{\omega}$ non-reacting shunt across the nerve, in the second with a 15830$^{\omega}$, in the others with a 23750$^{\omega}$. The axes of the coördinates on the tube are not quite rectangular necessitating a trigonometric correction in locating abscissae not on the X axis. The time in sigma of all of the records is indicated by marks on the base line of the lower record of the second column.

proximal lead. This curve could then be subtracted from the combined curve of shock and action current to obtain the shape of the latter. Even with this precaution there were residual inaccuracies due to the fact that the "escape" has a slightly different shape after the nerve is killed, as was mentioned in a previous paper. The data presented, unless qualified, concern only the α group. The records obtained, using an interval of 1.39σ, are presented in figure 3. Owing to the movement of the whole figure in a vertical direction, when the film is applied to the tube, the photographic records lose much of the original clearness of the figure. The data from these records and from a similar series, at an interval of 1.99σ, are included in table 2. The relation of the delay to the distance of conduction is plotted in figure 4 after the manner of figure 2. The data and the shape of the curves confirm the points indicated by

TABLE 2

DISTANCE CONDUCTED	INTERVAL 1.39σ					INTERVAL 1.99σ				
	Delay		Time to maximum		Delay crest of β	Delay		Time to maximum		Delay crest of β
	Start	Crest	Normal	Refractory		Start	Crest	Normal	Refractory	
mm.	σ	σ	σ	σ	σ	σ	σ	σ	σ	σ
12	0?	0.29	0.41	0.69		0?	0.07	0.42	0.52	
31	0.64	0.79	0.66	0.78		0.20	0.23	0.70	0.73	
46.5	0.72	0.86	0.74	0.89	1.13	0.33	0.40	0.76	0.84	0.81
62.5	0.78	0.94	0.79	0.95	1.15	0.36	0.46	0.75	0.84	0.78
82	0.82	1.10	0.80	1.08	1.44	0.42	0.48	0.80	0.87	0.92

corresponding data obtained from the phrenic nerve of the dog. The records also allow the determination of the positions of the crests of the β waves, and from these determinations, though crude, it can be seen that the progress of the β crests resembles that of the α crests, though at a given interval β is delayed to a greater extent than is α. Like that of α, the delay of β is less when the two stimuli are separated by the longer interval than it is when they are separated by the shorter one.

A glance at figures 2 and 4 shows that, particularly at the shorter intervals of stimulation, the delay of the second response is already considerable at the shorter distances of conduction. It is therefore necessary to determine what is occurring between the time of stimulation and the appearance of the response at the nearest lead. Several possibilities may be suggested: a delay in setting up of the second response under the electrode; a failure of the process to propagate itself until a finite time after it is set up; a very slow initial velocity which gradually increases; or a combination of these possibilities. The convergence of the curves of the positions of the starts and crests in figures 2 and 4 suggests that in the

extrapolation of the curves, the convergence will continue. The abrupt swing of the curves toward the Y axis in figure 4 suggests that the curves in figure 2 will take the same course; this is indicated by the extrapolation with dotted lines. Evidence for the validity of this extrapolation may be adduced from experiments. In the first place it may be shown that under the conditions of our experiments the second process seems to be set up without delay, and to have the same duration of the rising phase as the first.

THE DEVELOPMENT OF A SECOND RESPONSE UNDER THE STIMULATING ELECTRODE. The sciatic nerve of a green frog, *Rana pipiens*, was used. A non-polarizable electrode was placed on the nerve in such a position that it would be the cathode of the application of a break induction shock. (The anode was platinum and about 3 mm. central.) The same electrode was also connected to the recording apparatus, the other lead being from the killed end. The stimulating electrodes were connected to the two induction coils so that the second stimulus could be applied early in the relatively refractory period of a first. The second stimulus was just super maximal for the normal nerve; the first stimulus was slightly stronger than the second. The method of obtaining the curves and of their analysis can be followed by reference to figure 5.

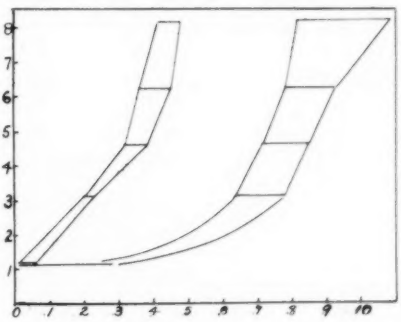


Fig. 4. Graph from the data of table 2. Abscissae: Delay in sigma. Ordinates: Distance, in centimeters, which the impulse had traveled when the reading was made. In the first pair of curves the interval between the stimuli is 1.99σ, in the second pair 1.39σ. The first curve of each pair shows the delay of the starts, the second the delay of the crests.

The curve *A* is a "second" action current, not preceded by a first, superimposed upon the escape. The curve *B* is the action current from the same stimulus when the nerve is partially refractory; the action current from the first stimulus plays no part in determining the form of this curve. To get the form of the "escape," the interval between the first and second stimuli was selected so as to make the second stimulus fall in the absolutely refractory period of the first. This gave the curve *C*, but the first action current *D* now appeared in the record. The form of the "escape" is therefore obtained by subtracting *D* from *C*, which gives the curve *E*. Now by subtracting *E* from *A* and *B* respectively, the form of the two action currents may be roughly ascertained. For the actual per-

formance of these operations the original tracing was photographed, then enlarged, the print being made through a glass plate ruled with millimeter coördinates. The curve resulting from the analysis was then again reduced to the scale of the original tracing. The result shows no detectable differences between the times of start and the durations of the rising phases under the two conditions, the action current in the partially refractory nerve, *F*, differing from that in the normal, *G*, only in its smaller size.[1]

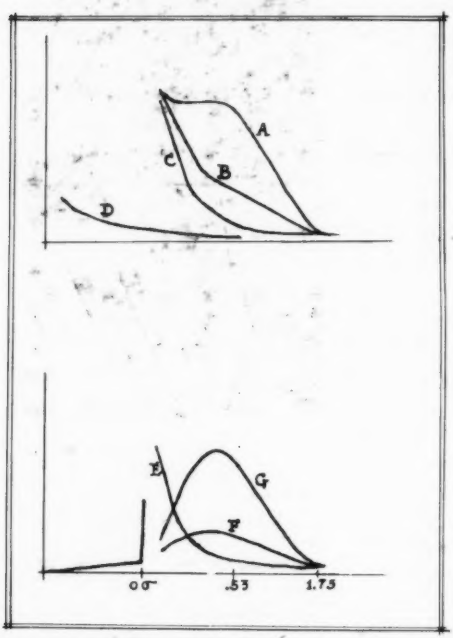


Fig. 5. The form of the action current of a process in partially refractory nerve when led from the stimulating cathode.

Upper: Reproduction of tracings made on screen of the Braun tube.

Lower: The action currents obtained by analysis of the tracings. 1 mf., 3000ω. Shunted by 11870ω. Time in sigma. Natural size.

To avoid so large an "escape," records were made from the sciatic nerve of another green frog in which the proximal lead was 1 mm. from the stimulating cathode. In this case also the only difference revealed by the two curves is a lower potential of the second process (fig. 6).

CONDUCTION OF A SECOND RESPONSE NEAR ITS ORIGIN. The preceding experiments indicate that there is no delay in the setting up the second process under the stimulating electrode; in the following experiment it will be shown that there is apparently no delay in the commencement of its propagation. This experiment was also performed on the sciatic nerve of the green frog, on account of the smaller size of the "escape," and the smaller spread of the stimulus, which render it possible to make leads from the nerve in closer proximity to the stimulating electrode. The records were made with a very rapid deflection of the elec-

[1] The conclusion reached in this section should be reserved to the condition of the experiment: stimulation by a rapid induction shock having a utilization period immeasurably short.

tron stream along the abscissa to increase the accuracy of the measurement. Some of the curves are reproduced in figure 7. In addition to those reproduced, records were made of the first action current alone, to aid in the location of the start of the action current in the partially refractory nerve. The data are abstracted in the legend of figure 8, which is a graph of the delay of the starts and crests.

The delay of the start of the second process is very slight at 5 mm. of conduction, but is much more at 15 mm. It continues in the interval between 15 mm. and 25 mm., though at a decreasing rate. The delay curve (fig. 8) indicates that the wave begins to propagate itself at once, otherwise the extrapolated curve would have to cross the X axis. As a matter of fact it crossed the Y axis at about 3 mm. Aside from error in measurement this probably represents the spread of the stimulus. In the bullfrog nerve (fig. 4) where the spread of the stimulus is known to be greater, the curve crosses the Y axis at 1.2 cm. In this case there must be some error of observation, however, as the crest is delayed while the start is not. The stimulus could not have spread 1.2 cm. or the crest would not be delayed; propagation having occurred, the delay of the fastest wave could not have been zero or it would have remained so at the other distances. The delay of the crest in the part of the nerve close to the point of stimulation will be discussed later.

THE VELOCITY OF THE SECOND OF TWO DISTURBANCES. A study of the delays of the starts of the waves, that is, of the delays in the times of arrival of the fastest elements under the leading-off electrode, shows a behavior more regular than that of the crests. In almost every case an exponential relationship may be found between the distance of conduction and the delay of the response, which holds fairly well, except in some experiments for points

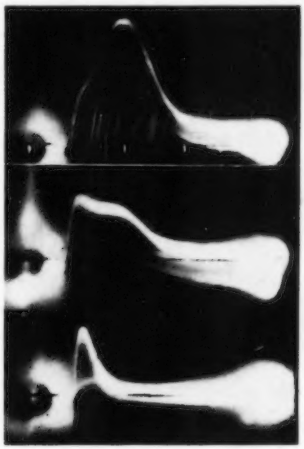
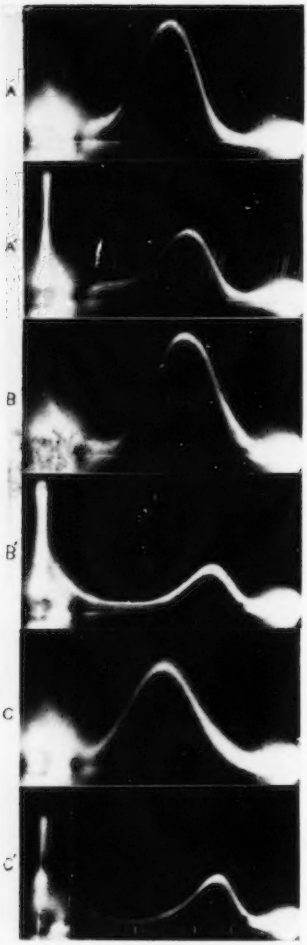


Fig. 6. The form of the action current of a process in partially refractory nerve; lead 1 mm. from the stimulating electrode. *Rana pipiens*. 1 mf., 2000$^{\omega}$. Nerve resistance 71,000$^{\omega}$ with a 9,500$^{\omega}$ shunt. The action current is therefore about 1/10.5 of the size it would have on direct input. Reproduced $\times \frac{1}{2}$.

Upper curve: "Second" action current alone; time indicated in sigma.

Middle curve: Made under the same conditions except that the wave was preceded by another produced by a stimulus 1.6σ earlier (absolutely refractory period ends 1.19σ).

Lower curve: Escape of the second stimulus obtained by killing the nerve between the stimulating electrodes and lead. The first action current was so far to the right that it did not affect the location of position of the crest.



close to the stimulating electrodes. As such a relation is entirely empirical, it would be unwarranted to attempt to formulate from it any law of conduction. Its sole value is in extrapolation of the curve, and in affording a means of visualization of the velocity curve of the second disturbance. The general form of the relation between the delay of the second response x and distance of conduction y, is $y = C\,10^{mx}$. The normal velocity of conduction is generally supposed to be linear, and the recent experiments of Bishop, Erlanger and Gasser (unpublished) demonstrate this to be the case. This relationship may be expressed by $x = \frac{y}{A}$: i.e., the time necessary for the impulse to reach any point is a constant function $\frac{1}{A}$ of the distance y. For the second of two impulses to reach any point in a refractory nerve, it will take the normal conduction time plus the delay of the second impulse in reaching that point. Therefore the expression connecting time and distance is $t = \frac{y}{A} + \frac{1}{m} \log_{10} \frac{y}{C}$. The slope of this curve gives the velocity, and can be obtained by differentiation with respect to time. As a result an expression is obtained of the form velocity $= \frac{1}{\frac{1}{A} + \frac{B}{y}}$,

Fig. 7. Action currents from the sciatic nerve of the green frog. 1 mf. 1000^{ω}. The upper curve of each pair is the "second" action current not preceded by a first; the lower curve is the action current produced by the same stimulus when it is preceded by another 1.78σ earlier. Records of the first action current alone were taken but are not reproduced. Time in sigma of all of the records is indicated on base line of the last.

Upper pair at 5 mm. conduction distance. $9{,}500^{\omega}$ shunt across nerve.

Middle pair at 15 mm. conduction distance. $15{,}830^{\omega}$ shunt across nerve.

Lower pair at 24.5 mm. conduction distance. $15{,}830^{\omega}$ shunt across nerve.

Axes of this tube slightly oblique demanding a trigonometric correction. Reproduction $\times \frac{1}{2}$.

where A is the constant of normal conduction and B is another constant whose value is $\frac{0.434}{m}$. It follows from this expression that conduction becomes normal when y becomes very large with respect to B. In fact, in the case of the phrenic nerve cited above, when the stimulation interval was 0.90σ, the velocity would have become 99 per cent of normal, after the impulse had travelled 28 cm. In the frog nerve it would have required 89 cm., for the interval: 1.39σ. The velocity curve for the latter is plotted in figure 9.

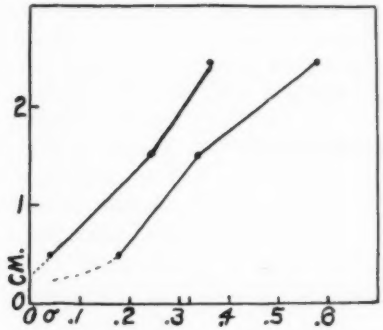


Fig. 8. Graph of data from figure 7 plotted as in figures 2 and 4.
Abscissae: Delay of starts and crests in σ. Ordinates: Distance of conduction in cm.

RECORDS	DISTANCE OF CONDUCTION	DELAY OF WAVES IN THE RELATIVELY REFRACTORY PERIOD BEHIND THE POSITION OF THE CORRESPONDING NORMAL WAVE		TIME TO MAXIMUM	
				Normal	Refractory
	mm.	σ	σ	σ	σ
A and A′	5.0	0.04	0.18	0.43	0.58
B and B′	15.0	0.24	0.34	0.51	0.61
C and C′	24.5	0.36	0.58	0.51	0.68

A part of this velocity curve depends upon the accuracy of the formula of extrapolation. For the length of the nerve, except at the very start, the same result could have been obtained graphically from the data, and is therefore independent of the formula. On the other hand, the value for the length of nerve over which the impulse must travel to reach a normal velocity, depends upon the accuracy of the formula; but in any case a consideration of the data shows that they at least illustrate the fact, that the necessary distance is much longer than the nerve under observation. The end of the relatively refractory phase may be determined either as the separation of two induction shocks at which the second has the same threshold value as the first, or at which the second response has the same

velocity of conduction as the first. The two values agree. For instance, in one bullfrog nerve in which the experimental conditions were similar to those here recorded, the relatively refractory period lasted 8σ. As the separation of the two processes was but 2.1σ at 8.2 cm. in the bullfrog nerve of figure 3, and since the rate of lag per centimeter had become small, and the velocity was already about 80 per cent of normal, it is obvious that the impulse would have to travel a considerable distance before it could work itself free from relatively refractory nerve.

The curve of figure 9 is drawn as though the initial velocity were a very small quantity. Due to the disturbing factors, such as spread of stimulus and difficulty of measurement of records made close to the stimulus, it is impossible to give the initial velocity a precise value, and the empirical extrapolation is not known to hold near the stimulus. Considering that there is no delay in setting up the process under the electrode, that the early velocities are slower the shorter the interval of stimulation, and that the normal disturbance presumably starts to propagate itself immediately at full velocity, we may picture, according to the interval of stimulation, a whole series of initial velocities ranging from near zero for short intervals to normal for infinite separation. This statement is supported by the observation that, in a single nerve, if at various intervals of separation of stimuli the curves of delay are plotted in such a way that the start of a curve for a longer interval is placed on that for a shorter interval at the point on the latter curve where the two processes are separated by conduction to the extent of the separation of the stimuli in the former, all thepoints lie on the same curve.

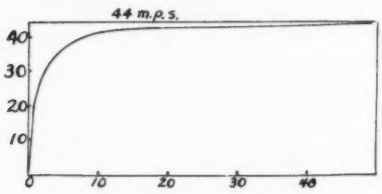


Fig. 9. Curve connecting velocity of conduction of a disturbance in a partially refractory frog nerve with the distance it has traveled. It is the graph of the expression, velocity $= \dfrac{1}{0.227 + \dfrac{0.206}{Y}}$.

Abscissae: Distance of conduction. Ordinates: Velocity in meters per second.

Discussion. *The cause of the delay:* All the evidence in this research points to a subnormal rate of transmission as the sole cause of the delay. Forbes, Ray and Griffith decided from their experiments that this was not a sufficient explanation. While they found that the interval of response might be double that of the stimuli at a distance of 2 cm. from the point of stimulation, they found that the further delay was rather slight. They assumed, to explain the early delay, that instead of a very small initial conduction rate, there was a delay in the inauguration of the response of the nerve to the stimulus, until the nerve had recovered to a point where it could respond to a persistent local effect resulting from the

exciting current. The abrupt swing of the delay curves toward the Y axis in figures 4 and 8 and the appearance of the second process under the stimulating electrode without delay, show that a great initial delay can be explained by slow conduction (Erlanger, Gasser and Bishop, 1924). On the other hand, the possibility cannot be denied that, under special circumstances, with early excitation, the tissue might respond after further recovery to a remainder of the exciting stimulus, the recovery time becoming thereby an apparent cause of delay; but if we adhere strictly to the definition of the absolutely refractory period, as the period in which the nerve is unable to respond again, then the second stimulus in this case is falling within the absolutely refractory period.

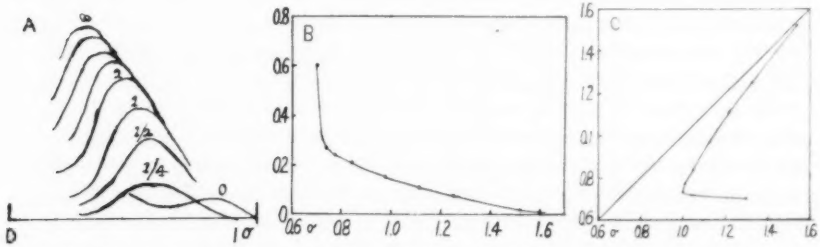


Fig. 10. *A*. Dog phrenic at 37°C. Showing delay of a second response when its stimulus is preceded by another stimulus at the indicated intervals. 1 mf., 1000Ω. Position of end of first sigma marked. Reproduced × 0.86.

Intervals of stimulation marked in number of turns of screw controlling position of floating arm. The values are as follows:

∞	no previous stimulus	1	0.84σ
6	1.52σ	½	0.77σ
4	1.25σ	¼	0.73σ
3	1.11σ	0	0.70σ
2	0.97σ		

B. Delay of the crests (abscissa) plotted against the stimulation interval (ordinate).

C. Interval of response (abscissa) plotted against interval of stimulation (ordinate).

They also mention another phenomenon which cannot be explained on the basis of delay alone. Lucas described a curve similar to 10, *C*, for the sciatic-gastrocnemius preparation of the frog. Forbes, Ray and Griffith showed that this type of curve could be obtained from the nerve alone. We have not many data suitable for plotting according to the Lucas schema, but one experiment on a dog phrenic, which had been in the incubator at 37° for 7 hours, confirms the observation of Forbes, Ray and Griffith. In this experiment, at the constant distance of conduction of 1.5 cm., the positions of the responses to a stimulus at a constant position

were recorded, when preceded at various intervals by another response. The result is shown in figure 10, *A*. While, as has been mentioned, the positions of the crests are not an exact index of the delay of the wave, the error is not sufficient to interfere with this argument, and the delay of the crests is plotted against the interval of stimulation in figure 10, *B*. The same data are replotted according to the Lucas schema in figure 10, *C*, and the curve bends away from the Y axis at the start. In other experiments plotted in this manner the bend does not appear. Forbes, Ray and Griffith expressed the opinion that this bending back of the curve cannot be explained on the basis of slowed conduction, while it harmonizes perfectly with the idea of persistence of the local excitatory process.

We may examine the above experiment, in the first place to see if the curve cannot be explained on the basis of slow conduction, and in the second place to determine the probability of its being due to a delay of the original response. A comparison of parts *B* and *C* of figure 10 shows that, when the effect of the decrement of interval of stimulation is to produce a disturbance early enough to be conducted so slowly that the increment of delay is greater than the decrement of interval, in other words, when the tangent to the delay-interval curve passes through the absolute value of one, the curve connecting the interval of response to the interval of stimulation will show the designated bend. At the start of the curve, a decrease in the interval of stimulation of 0.03σ caused an increase of delay of 0.34σ. It is inconceivable, if the nerve responds immediately to a second stimulus 0.73σ after the first, that it would not respond until about 1.0σ after the first, when the stimulation interval is 0.7σ. Some other explanation is necessary, and slow conduction is not only a possible one, but it is also the only probable one.

The potential of the action currents: The effect of the slower velocity of a disturbance propagated in a nerve rendered refractory by previous activity, is to increase the distance between the two processes, with the result that the second one progressively works itself into a more advantageous position, so that finally, if the nerve were long enough, it would find itself in nerve fully recovered. It should then have all the properties of a normal disturbance. Therefore, if the small response, produced by a second stimulus early in the relatively refractory phase, be due to a low grade of activity in all the fibers, then, as the process reaches a more advantageous position, it should grow in area and ultimately reach normal. An examination of the data shows that this is not the case.

In the experiment recorded in figure 7 the second disturbance first decreased in size, then increased. In this figure it should be noted that the record at the 5 mm. lead was made with a greater shunt than the other two, therefore the processes in the refractory nerve can be compared as to size only with the corresponding normal process and not with one

another. Their absolute potential cannot be given as the resistance of the nerve was not measured. In conduction from the 5 mm. lead to the 15 mm. one, the processes in both the normal and refractory nerve decreased in size, but the latter more rapidly. Although the shunt was smaller for the 15 mm. records, the recorded potential of the normal process was only about the same as at 5 mm., while that of the process in the refractory nerve was smaller. In passing through the succeeding centimeter (the records were made under the same conditions), the process in the refractory nerve increased in size in spite of the usual conduction decrease in the normal process.

In the phrenic nerve of the dog, described in figure 1 and table 1, both processes decreased in size, but the second more rapidly. Its rate of decrease was greater when the first and second stimuli were close together than when they were farther apart: in other words, there was a relation between the decrease in size and the degree to which the nerve, in which it was being propagated, was refractory.

In some other phrenic nerves examined the state was similar to that in figure 1, in others a tendency for the second process to grow could be seen. For instance, in a nerve whose normal action current decreased but little on conduction and in which the second stimulus was applied early in the refractory phase, so that the second process was initially very small (the absolutely refractory phase ended after 0.58σ, the interval between the stimuli was 0.66σ, velocity of conduction 57 m.p.s.), the size of the second action current increased from 21 per cent of the corresponding normal at 18 mm. of conduction to 38 per cent at 82 mm.

In the bullfrog sciatic nerves of figures 3 and 11 the size of the second process increased from the start, in the latter but slightly, in the former from 20 per cent of normal at 3.1 cm. to 45 per cent of normal at 8.2 cm. Although the velocity had increased to 90 per cent of normal, the recorded potential had less than 50 per cent recovered.

It is thus apparent that, according to the conditions of the experiment, there may occur a progressive increase of the second process, a progressive decrease, or a decrease followed by an increase. An increase can readily be explained on the basis of entrance of the disturbance into less refractory nerve. But it should be noted that the recovery of potential does not correspond to the recovery of velocity; either other factors, tending to decrease the height, counterbalance this recovery, or all the fibers in the nerve do not originally respond to the second stimulus.

To explain the decrease in size, it is necessary to consider the causes of the decrease in so-called normal nerve, since the difference appears to be only one of degree. These have been found by Bishop, Erlanger and Gasser (unpublished) to be of two sorts: one operating only in the vicinity of the stimulus; the other, due to the differences of the conduction rate

in the individual fibers, acting to the same extent throughout the nerve. The result of these two factors is that the potential decreases more rapidly at first: which is the only condition necessary for the explanation of all the cases described above.

In the phrenic nerve showing progressive decrease in size of the second process, the latter is initially large and has little opportunity to increase; therefore, only the factors decreasing the size have a visible effect. But this effect is greater than in normal nerve. In the bullfrog sciatic the second disturbance is initially small, and its tendency to grow more than counterbalances, at all stages, the opposing influences. In the green frog nerve, initially, during the period of most rapid decrease in potential the growth of the process is more than counterbalanced by the combined effect of the two factors producing reduction, but later, when out of the range of one of the factors, the net result is an increase in the size of the disturbance.

The duration of the rising phase of a process in partially refractory nerve has been shown to be longer at every distance of conduction than that of the corresponding normal process; the durations of both increase with the distance of conduction, but that of the former to a greater extent. In explanation of this phenomenon, it should first be recalled that the action current does not represent the nature of the change in any one nerve fiber, but is compound. It is an algebraic summation of processes not always in phase, and in a preliminary paper it has been shown that the increase in the duration of the rising phase of the normal process may be satisfactorily explained by slight differences in conduction rates in the constituent nerve fibers (Erlanger, Bishop and Gasser, 1925). In the partially refractory nerve the greater prolongation of the rising phase might be due either to a greater effect of those factors operating on the normal process to prolong its rising phase on conduction, or to a prolongation of the process in each individual fiber.

There does not seem to be anything about the refractory state of the nerve fiber, which of itself increases the duration of the rising phase, otherwise it would be longer when a lead is made from the stimulating electrode. That during propagation the process may be prolonged is also highly improbable. A prolongation of the wave is not a property that has been ascribed to conduction with a decrement and the very existence of this type of conduction seems to be disproved by Kato (1924) and by Forbes (1925) and their colleagues. The theory must therefore be advanced as most probable that the constituent waves become mutually separated to a greater extent than in normal nerve, that is, that the refractory state produces a greater variation of the velocities of conduction in the different fibers. A basis for this may be found in a consideration of the refractory periods of the different fibers and of the form of the

velocity curve of the second response. It was shown in a previous paper (Erlanger, Gasser and Bishop, 1924) that the slower fibers in the nerve trunk have a longer refractory period than the faster ones; therefore when all the fibers are stimulated at once, the stimulation falls in different positions in their relatively refractory phases and the process in the slower fibers starts to be propagated at a rate slower relative to the normal velocity in the fiber than obtains in the faster fibers. The action current thereby is more rapidly prolonged.

When the interval between the two stimuli is short and a lead is made at a short distance from the stimulating cathode, the time to maximum increases very rapidly on conduction between the stimulus and the lead (figs. 4 and 8). The shape of the right hand curve in figure 4 shows how slow the conduction is in the fastest fibers when the interval of stimulation is short. For the other fibers the stimulus must fall closer and closer to the absolutely refractory period, probably finding some fibers even absolutely refractory. The conduction in these fibers will be very slow indeed, causing a much greater delay with respect to the fastest fibers, and thereby a greater rate of increase in the duration of the rising phase than when the velocities later increase and approach their normal values which determine the normal rate of prolongation of the wave.

The increased duration of the wave in partially refractory nerve, as compared with a normal wave in a similar position, would account for a part of the failure of a wave emerging from partially refractory nerve to gain the full potential of the normal wave, but it is not sufficient. If we again examine the experiment of figure 3, in which the potential recovered to about 45 per cent of normal, when the calculated velocity was 90 per cent of normal, we find the rising phases have the ratio $\frac{1.08}{0.80}$. A simple geometrical construction, made according to the theory of separating constituent waves, shows that the increased length of the wave can account for but a small portion of the discrepancy; the conclusion, therefore, becomes natural that originally, if the second stimulus fall early enough, all the fibers are not stimulated as some of them are still absolutely refractory.

Stimulus not a cause of delay. Previous evidence seems to show quite clearly that the delayed conduction in relatively refractory nerve is due to the disturbance which had previously been propagated, and not to local effects of the stimulating current. While Gotch (1910) established (by applying the two stimuli at different positions on the nerve) that the delay is due largely to the previous passage of an impulse, he thought that it is also due in part to a local fatigue caused by the stimulus, inasmuch as prolonged faradisation caused a marked change in potential and time of appearance of a single response. Keith Lucas (1911) criticised this opinion

on the basis that, while the experiment shows that a local fatigue can be produced by prolonged stimulation and thus cause a delay of the response, it does not follow that the fatigue, produced by a single stimulus, would have any appreciable magnitude compared with that produced by the propagated disturbance. He then quite clearly showed that in muscle, at least, the propagated disturbance is the only factor as, *a*, if a third stimulus is interpolated between the first and second and in the absolutely refractory period of the first, it is without effect on the second response; *b*, application of the second of two stimuli at a definite position in the disturbance, results in identical changes of the second response, whether the two stimuli are applied at the same or at different points on the nerve; *c*, the electrical response is not delayed in sartorius muscle when the stimulus is just preceded by a subminimal one; *d*, the delay of the response to the second stimulus occurs in the ventricle of the frog when the first response is normal.

Inasmuch as in our experiments the stimuli were repeated twenty to thirty times per second, and as other changes not previously described have been observed, an experiment was done in which the stimuli were applied at different points on the nerve. The duration of the refractory period was determined at a point 40 mm. distal to the first stimulus to be 1.22σ. This was done by determining the earliest stimulus applied at this point, which would just cause a second response to appear in a more distal lead. The action current produced by the first stimulus was then recorded at the point of stimulation by the second, together with the "escape" of the second stimulus (reduced in size), to indicate its position with respect to the first process (fig. 11). As the action current in nerve 40 mm. from the stimulus does not represent the process in any one fiber, one has a datum indicating merely the time after the arrival of the fastest wave, that the nerve as a whole, and presumably the fastest fibers, are capable of a second response. The interval of stimulation was then lengthened to 1.93σ, its length being determined by the same method as the refractory period. The form of the two responses was then followed through the rest of the nerve, non-polarizable electrodes being placed at

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Fig. 11. Action currents of a second process in the sciatic nerve of the bullfrog when the two stimuli are applied 4 cm. apart. 1 mf.; 2000^{ω}; nerve shunted by $23{,}750^{\omega}$. Time in σ for all indicated in base line of one of the records.

Upper row: second process alone; middle: second preceded by a first; third row: end of first process.

Column I, 2.1 cm. from second stimulus; II, 3.7 cm.; III, 5.6 cm. In lower left corner a record showing position of second stimulus 1.93σ after start of action current. The absolutely refractoryperiod determined by the same method ended at 1.22σ. Reproduction $\times$ 0.74. Graph: delay-distance curve in the manner of figures 2 and 4.

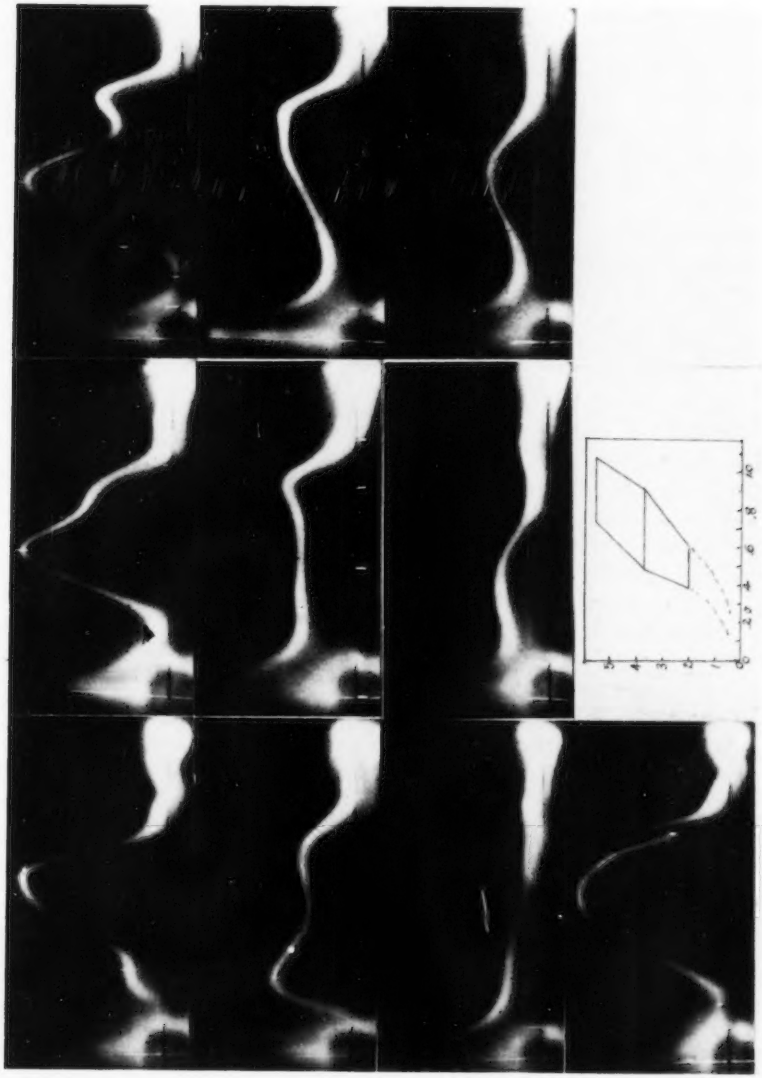

Fig. 11

21, 37 and 56 mm. from the second stimulus. These could each in turn be used as leads, the other lead being from the killed end. The customary records were made of the "second" process alone, and of the first process, to aid in the selection of the start of the second.

The records show the same effects that occur when the two stimuli are applied to the same point; the progressively greater delay of the start, and the increasing duration of the rising phase, so that the crests show a still greater lag.

SUMMARY

The mode of conduction of a second process, soon after a previous one, was studied in the frog's sciatic nerve at room temperature and in the phrenic nerve of the dog at 37°, in oxygen. There is great similarity in the two nerves.

In confirmation of Gotch and Keith Lucas, it was determined that there is a delay of the second process, which is greater the longer the distance of conduction and the shorter the interval between the stimuli.

The delay of the second process is greatest in the first few centimeters of conduction, but has been followed for 12 cm. By extrapolation of the curve it continues much farther.

When an induction shock of short duration is used as the stimulus, the second response develops under the stimulating cathode as promptly as the normal process. It has the same time values, but a lower potential.

An experiment on the sciatic nerve of the green frog showed that propagation starts without delay.

The observations show that the delay of the second response is due entirely to a slower rate of conduction. They thus support the explanation offered by Gotch.

From a simple exponential expression, derived empirically, relating the delay to the distance of conduction, it has been possible to calculate the velocity curve, and to plot this against the distance of conduction. The velocity rises to a large percentage of normal within 10 cm. The delay of the second process causes it to be transferred into more irritable nerve, where the velocity is greater, and the delay therefore decreasingly less per centimeter of conduction.

The duration of the rising phase is always larger in partially refractory nerve than the normal process at the same position. The rising phase of the normal process increases in duration on conduction, and this increase is greater in the repeated process, so the crest of the latter lags progressively more than the start.

If the second stimulus occur early in the relatively refractory period, the resulting disturbance is small and increases on conduction. A later stimulus produces a larger response, which may decrease in size on conduc-

tion, or initially decrease then increase. The increase is explained as due to greater potential developed in the individual fibers as the process emerges from refractory nerve. The decrease in potential, as well as the increased duration, is explained as due to the same factors which produce a decrease in size of the normal wave.

The mode of delay of the second response is the same, when the first and second stimuli are applied to different portions of the nerve, as when they are applied to the same portion (confirming Lucas). The disturbance also undergoes the same changes on conduction.

The beta wave is also delayed in partially refractory nerve, and to a greater extent than the alpha.

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CIRCULATORY RESPONSES TO STATIC AND DYNAMIC EXERCISE

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The observations reported in this paper fall into two groups; the first a series of eight experiments on seven subjects in which the arterial and venous pressures, pulse rate and respiratory rate were followed before, during and after a period of static exercise; the second a series of six experiments on two subjects in which the response of the venous pressure to light, moderate and severe dynamic exercise was followed. Those on static exercise may be considered first.

The purpose of the experiments is to follow the circulatory response to a period of isometric muscular contraction. In ordinary forms of muscular exercise one of the chief factors concerned in the return of the blood to the right heart is the propulsive action of the muscles themselves. It seemed worth while to observe the circulatory response to a form of exercise in which this action of the muscles was not present. Moritz (1918) observed the heart shadow with the Roentgen ray during static exercise and found it decreased. Exercise in his experiments consisted in holding the arms or legs extended to the point of fatigue, the subject lying on a table. In these experiments the pumping action of the skeletal muscles was absent but no observations were made on the arterial or venous pressure.

In our experiments the subjects were in all cases healthy males ranging in age from 22 to 30 years. The experimental procedure was as follows: The subject sat in a chair which was firmly bolted to a base and tilted slightly backward. His head was supported by an adjustable head rest and his arms by shelves on each side of the chair, the height of the shelves being adjustable. The legs were extended and supported in a nearly horizontal position by a foot rest; considerable care was exercised in seeing that the subject was in a comfortable position. The subject sat at rest 5 minutes after the pneumograph, sphygmomanometer cuffs and venous pressure capsule had been adjusted; the control or pre-exercise readings were then made. Arterial pressure determinations were made simultaneously by the Erlanger instrument and by the auscultatory method, the auscultatory systolic and diastolic criteria being electrically signalled on the same drum on which the graphic record was made. Venous pressure

was followed by a modification (White, 1924a) of Hooker's method (1914). After a few resting or control readings had been made the foot rest was

EXPLANATION OF TABLES 1 TO 8

Each table, 1 to 8, gives the data for an experiment. The subject's initial follows the table number. The control readings are above the first line, the readings made during work are between the first and second lines and the post-exercise readings are below the second line. V. P. signifies venous pressure in cm. water; Art. P. with subheads S and D signifies systolic and diastolic arterial pressure, respectively, in mm. Hg.; P. P. is pulse pressure; P. R., pulse rate; P. P. × P. R., their product, and R. R., respiratory rate.

TABLE 1

Mov.

MINUTES	V.P.	ART. P.		P.P.	P.R.	P.P. × P.R.	R.R.
		S.	D.				
						Graphic	
	8½	116	60	56	75	4200	24
	8½	118	62	56	76	4256	23
	8	116	60	56	74	4150	22
		118	66	52	75	3900	23
1	11	140	74	66	84	5564	23
2	12	138	72	66	81	5346	26
3	13½	130	70	60	83	4980	25
4	14	134	67	67	79	5293	23
5	14	140	74	66	87	5742	24
6	13	134	74	60	83	4980	23
7	12	138	84	54	84	4536	24
8	12½	134	82	52	84	4368	24
9	12½	148	80	68	84	5712	27
10	12½	144	82	62	81	5022	25
1	7½	128	60	68	75	5100	26
2	8½	120	60	60	75	4500	26
3	8	116	58	58	73	4234	24
4	8	114	55	59	75	4420	26
5	8½	114	57	57	72	4104	24
6	8½	112	53	59	75	4420	25
7	8½	108	53	55	75	4125	24
8	8	114	54	60	73	4380	24
9	8	108	51	57	74	4220	23
10	8½	110	53	57	74	4220	23

TABLE 2

R.

MINUTES	V.P.	ART. P.		P.P.	P.R.	P.P. × P.R.	R.R.
		S.	D.				
						Graphic	
	12½	98	68	30	78	2340	19
	12	102	63	39	75	2925	16
	12½	104	62	42	75	3150	19
	13	102		36	77	2772	20
	13	100		35	75	2625	20
1	11	112	72	40	81	3240	23
2	12	108	65	43	81	3483	22
3	11½	108	64	44	83	3652	21
4	12	110	61	49	85	4165	22
5	12½	114	70	44	90	3960	21
6	12½	114	63	51	90	4590	22
7	14	112	66	46	90	4140	23
8	12½	112	67	45	96	4320	27
9	13½	118	70	48	96	4608	24
10	14	114	66	48	93	4464	25
1	11	105	52	53	84	4452	15
2	12½	104	51	53	84	4452	17
3	12½	104	54	50	78	3900	20
4	13	105	52	53	75	3975	16
5	13	105	56	49	70	3430	16
6	12½	104	52	52	74	3848	16
7	13	102	51	51	75	3825	16
8	12	104	60	44	69	3036	18
9	12½	108	52	56	74	4144	19
10	12	106	52	54	70	3780	19
11	10½	104	50	54	72	3888	18
12	12½	108	50	58			
13	12½	102	50	52			

removed and the subject held his legs extended in a nearly horizontal position. This position was maintained for 10 minutes during which pe-

riod readings were made at intervals of 1 minute. The foot rest was then returned and the readings continued at 1 minute intervals for 10 to 15 minutes of the post-exercise period. The data of the various experiments are given in tables 1 to 8.

The immediate effect of a strong isometric contraction of a large proportion of the muscular tissue of both lower extremities is presumably to

TABLE 3

S.

MINUTES	V.P.	ART. P.		P.P.	P.R.	P.P. × P.R.	R.R.
		S.	D.				
	9½	99	50	48	69	3300	16
	8½	94	50	43	66	2840	17
	9	94	50	44	64	2820	16
1	7½	100	50	50	80	3960	20
2	8	106	54	52	78	4095	20
3	7½	108	60	48	75	3637	19
4	8½	117	60	58	76	4408	24
5	8½	110	64	45	78	3510	20
6	7½	115	60	55	81	4450	19
7	8	116	60	53	78	4130	15
8	8	104	56	47	81	3810	19
9	7½	104	62	42	88	3740	15
10	8	108	64	43	90	3870	20
1	7½	95	52	43	70	2980	15
2	8	100	52	48	73	3430	17
3	7½	99	50	49	69	3380	15
4	7½	92	52	40	66	2640	14
5	8	88	52	36	66	2380	12
6	7	88	51	37	66	2400	15
7	8	98			69		12
8	7	94	50	42	66	2772	16
9	8	100	50	48	69	3320	16
10	7	88	50	37	64	2370	14
11	7	90	50	39	67	2610	17
12	7½	92	50	42	62	2600	15
13	7½	88	50	38	67	2580	12
14	7½	90	50	40	68	2720	11
15	7½	97	48	48	66	3170	12

TABLE 4

Miy.

MINUTES	V.P.	ART. P.		P.P.	P.R.	P.P. × P.R.	R.R.
		S.	D.				
	12	106	69	37	69	2560	24
		106	66	40	69	2760	24
	12	100	64	36	67	2510	22
1	13	112	72	40	76	3040	25
2	13	118	82	36	83	2990	26
3	13½	124	68	56	81	4540	24
4	13½	112	74	38	78	2960	26
5	14	122	76	56	78	4370	25
6	14	122	82	40	78	3120	25
7	14	126	74	52	80	4160	24
8	15½	128	71	57	81	4620	24
9	16	126	68	58	75	4350	25
10	15½	122	63	59	78	4600	30
1	11½	107	60	47	67	3150	23
2	11½	108	57	51	65	3300	22
3	11½	104	56	48	66	3170	22
4	12½	106	60	46	65	2990	21
5	12½	104	57	47	66	3100	21
6	13	106	55	51	66	3370	21
7	12½	98	52	46	66	3040	20
8	12½	96	56	40	65	2600	21
9	12	100	52	48	64	3070	21
10	12	100	54	46	57	3080	21

squeeze out a large part of the blood. In other words, we are suddenly decreasing the circulatory bed without a corresponding decrease in the amount of circulating blood. The immediate effect of this factor should tend to increase both arterial and venous pressure. At the same time great care must be taken that the pressure relationships are not disturbed by changes in the intrathoracic pressure. In some subjects the venous

pressure mounted immediately on removal of the foot rest to 25 to 30 cm. of water. These subjects were unconsciously raising the intrathoracic pressure. This increased intrathoracic pressure was of course not continuous, as respiration continued and the pneumogram did not differ from that obtained on subjects who did not exhibit such a rise. It is probable that the laryngeal passage in such subjects is contracted during expira-

TABLE 5

Dav.

MINUTES	V.P.	ART. P. S	ART. P. D	P.P.	P.R.	P.P. × P.R.	R.R.
	8½	102	58	44	63	2770	15
	8½	102	58	44	63	2770	15
1	9½	114	60	54	75	4050	16
2	10½	122	62	60	84	5040	18
3	11	126	68	58	81	4700	17
4	11	132	75	57	83	4710	17
5	11	130	70	60	76	4560	16
6	10½	138			80		18
7	11½	136	76	60	81	4860	18
8	11	134	70	64	87	5570	19
9	10	138			87		20
10	10	142	76	66	85	5610	19
1	9	116			69		15
2	8½	110	52	58	63	3650	15
3	8½	106	56	50	63	3150	16
4	8½	102	52	50	62	3100	15
5	8½	108	52	56	66	3700	15
6	8	108	58	50	63	3150	16
7	8½	103	50	53	63	3340	15
8	8½	105	56	49	61	2990	17
9	8½		55		61		15
10	8½	104			66		15

TABLE 6

Dav.

MINUTES	V.P.	ART. P. S	ART. P. D	P.P.	P.R.	P.P. × P.R.	R.R.
	9½	98	62	36	63	2270	16
		95	56	37	65	2400	17
	10	95			64		17
	10	88	52	38	64	2430	18
1	9½	104	64	41	81	3320	18
2	9½	111	62	49	82	4020	20
3	10	116	56	55	82	4510	17
4	11	112	63	59	84	4960	19
5	11	118	60	58	82	4760	20
6	10	117	60	57	83	4730	20
7	11	116	65	51	87	4440	18
8	10½	118	65	53	90	4770	18
9	10½	122	72	50	88	4400	18
10	10						
1	8½	108	55	53	75	3980	18
2	9½	102	52	50	66	3300	16
3	10	99	57	42	66	2770	17
4							
5							
6	10	90	52	38	64	2430	16
7	10	94	54	42	67	2810	17
8	9½	90	58	34	64	2180	17
9	10	99	57	42	65	2730	17
10	9½						
11	10½	102	55	47	63	2960	16
12	10½	101	51	50	63	3150	17

tion so that the mean intrathoracic pressure is raised although the pressure falls below atmospheric during inspiration. Some subjects appear to be unable to breath normally, as evidenced by the great rise of venous pressure, while performing this type of exercise. Such subjects were not used for these experiments. It is quite probable that the decrease in heart shadow observed by Moritz during static exercise was due at least in part to an increased intrathoracic pressure. While one cannot say with

certainty that the intrathoracic pressure relations were normal during exercise in any of these cases, the assumption has been made that they were in those cases where the venous pressure does not show an immediate rise of more than 3 cm. of water. The subjects were all medical students who appreciated the importance of avoiding the performance of the Valsalva

TABLE 7

H.

MINUTES	V.P.	ART. P.		P.P.	P.R.	P.P. × P.R.	R.R.
		S	D				
	11	130	56	74	54	4000	18
	11½	130	58	72	54	3890	18
	11	130	62	68	56	3810	16
1	12½	128	70	58	72	4170	18
2	12½	138	64	74	72	5320	20
3	12½	146	71	75	69	5170	19
4	12½	156	66	90	72	6480	21
5	13	150	74	76	78	5920	20
6	13	146	78	68	78	5300	18
7	12½	152	74	78	81	6300	18
8	13	154	82	72	84	6040	19
9	13	154	82	72	86	6200	18
10							
1	10½	138	67	71	63	4470	20
2	11½	130	62	68	62	4210	16
3	11	122	58	64	54	3450	20
4	11	118	54	64	57	3650	15
5	11½	110			54		16
6	10	108	56	52	57	2970	19
7	11						
8	10½						
9	11						
10	11	116	58	58			
11	10½						15
12	11	112	58	54	54	2920	15
13	10	116	56	60	58	3480	15
14	10½	116	54	62	60	3720	19

TABLE 8

O.

MINUTES	V.P.	ART. P.		P.P.	P.R.	P.P. × P.R.	R.R.
		S	D				
	8	110	74	36	69	2480	16
		112	68	44	72	3170	15
	8	110	66	44	70	3080	16
		107	72	35	74	2590	10
	8	98	62	36	74	2660	11
1	9	120	66	54	80	4320	18
2	9	114	63	51	82	4180	21
3	9½	114	65	49	81	3970	20
4	9	112	60	52	83	4310	20
5	9½	124	68	56	87	4870	21
6	8½	118	70	48	87	4180	21
7	8	122	73	49	93	4560	23
8	9½	128	67	61	87	5300	22
9	9½	120	76	44	90	3960	21
10	9½	124	70	54	96	5190	21
1	8½	100	58	42	78	3280	17
2	9½	94	54	40	80	3200	18
3	8½	92	54	38	78	2960	20
4	9	96	50	46	70	3220	17
5	8	98	52	46	75	3450	18
6	8½	96	50	46	75	3450	18
7	8	96	48	48	72	3460	17
8	7½	104	50	54	68	3680	17

experiment. So many factors may affect the circulatory response to this type of exercise that it is impossible to predict what might be expected to happen. An inspection of the data shows that the responses are far from uniform. The subject Dav. (tables 1 and 2) on whom two experiments were performed showed practically no change in venous pressure during exercise in the first experiment and an unmistakable rise in the second.

Objective evidence that this exercise is really work is seen in the invariable rise of pulse and respiratory rates and arterial pressure. Subjective evidence any one may readily obtain for himself.

Some of the main points of the data may be briefly stated here. The venous pressure usually but not invariably exhibits a moderate rise (2 to 4 cm. water) during exercise. The maximum of this rise is usually not attained immediately. On cessation of exercise the venous pressure falls immediately to the resting level. The pulse rate invariably shows a significant rise which follows the usual return curve; the respiratory rate is moderately increased. The systolic arterial pressure shows a marked rise during exercise; this sometimes reaches its maximum immediately on beginning exercise but more often is progressive. The fall to normal is quite rapid, the control level usually being reached at the first reading, although this is not invariable. There is no tendency to a secondary rise immediately following the fall to normal such as has been recorded as a sequel of dynamic muscular exercise (Cotton, Lewis and Rapport, 1917). There is certainly no inverse relation between arterial and venous pressure changes. The behavior of the diastolic arterial pressure during static exercise is quite variable. There is sometimes a slight rise, sometimes a slight fall, sometimes no significant change. In two cases, Mov. and H., there is a rather marked and progressive rise. In every case there is an immediate fall on cessation of exercise even though the control level had been reached before exercise stopped. The pulse pressure practically always shows a significant rise which may or may not be progressive. The value of pulse pressure $\times$ pulse rate is given as an indication of the volume output of the heart. It is seen to rise markedly during exercise in practically every case, to drop sharply on cessation of exercise, and usually to show a progressive further drop to the control figures. The increased value of pulse pressure $\times$ pulse rate during static exercise indicates that the pumping action of the skeletal muscles is not an essential factor in bringing about an increased venous return. As to the factors which may operate during this type of exercise to effect an increased venous return, an increased respiratory action of the thorax is undoubtedly present. Whether or not this alone would suffice cannot be answered with certainty. It seems likely that some other factor must be operating and the data appear to offer at least indirect evidence in favor of the view advanced by Danzer (1925) that there is a peripheral vascular dynamic mechanism which aids in the return of blood to the heart; the effect of this mechanism is increased on exercise.

One might expect the sudden expulsion of blood from the isometrically contracted muscles into the venous system to result in an immediate rise in venous pressure. In most cases however the first venous pressure reading, which was usually made within 15 seconds after the isometric contrac-

tions began, shows little or no increase over the control figures. Apparently the increase in the volume of blood contained in the venous system is taken care of by a compensatory process the various factors of which are discussed under the dynamic exercise experiments.

To conserve space the arterial pressure determinations made by the auscultatory method have been omitted from the tables. They agreed well with the graphic readings. It may be noted that the resting figures for venous pressure given in the present tables are higher than those reported in a previous paper (White, 1924a). The reason for this is that in the present series of experiments the "systolic" venous pressure has been taken, i.e., the pressure required to abolish all pulsation in the vein, while in the previous set of experiments the "diastolic" venous pressure was taken, i.e., pressure required to produce collapse of the vein between pulsations. At that time we felt that the "diastolic" criterion was the most satisfactory. Subsequent experience has shown us the difficulty of deciding just what pressure is the one that produces complete collapse between pulsations. The establishment of the point at which all pulsations are abolished is much more nearly free from subjective errors. As our experience increases we find an increasing percentage of normal resting subjects exhibit a centripetal venous pulse at ordinary room temperatures. We also find that a higher pressure is required to abolish completely the venous pulsations than we were finding a year ago. The statement made in a previous paper (White, 1925) that "the difference between systolic venous pressure and diastolic venous pressure is at the most only a few millimeters of water" is certainly not true.

In the second set of experiments reported here the response of the venous pressure and pulse rate to dynamic muscular exercise of various degrees of severity was followed. Exercise was taken on the bicycle ergometer. The hand resting on an adjustable shelf was brought to the heart level and the subject allowed to sit on the bicycle for 5 minutes before the control readings were made. After the control readings the work of pedalling was started and continued for 30 minutes, venous pressure readings being taken every minute and pulse readings about every other minute. On cessation of exercise the readings were continued for 15 minutes. Three experiments were performed on each of 2 subjects, each subject engaging in a period of light, moderate and heavy exercise. Since facilities for measuring the absolute amount of work were not available the brake band was set at the same point for the whole series of experiments and the rate of work was judged by the speed of pedalling. Forty-eight to 50 r.p.m. was found to be light exercise, 60 to 70 moderate, and 85 to 90 heavy. The response of the venous pressure and pulse rate to bicycle pedalling at light, moderate and heavy rates in the two subjects is seen in table 9.

These data may be briefly discussed in the light of a hypothesis pre-

TABLE 9

D.									M.								
48 r.p.m.			65 r.p.m.			86 r.p.m.			50 r.p.m.			70 r.p.m.			90 r.p.m.		
Minutes	V.P.	P.R.	Minutes	V.P.	P.R.	Minutes	V.P.	P.R.	Minutes	V.P.	P.R.	Minutes	V.P.	P.R.	Minutes	V.P.	P.R.
	13	86		14	90		14	82		11	75		12	104		14	100
	13	88		14	88		14	84		10	72		11	102		14	100
	13½	88		14	88		15	84		11	72		12	102		13	96
1	17		1	16		1	17	116	1	20	96	1	20	126	1	27	140
2	18		2	16		2	16½		2	21		2	22	130	2	28	140
3	14		3	15½		3	16½	124	3	21	102	3	20	130	3	29	140
4	13	100	4	16	116	4	17		4	21		4	22		4	31	140
5	12	104	5	16		5	19		5	20	99	5	21	130	5	31	
6	11½		6	16½		6	19	120	6	19		6	21		6	32	140
7	11		7	15	118	7	20		7	17	99	7	19		7	32	
8	12	104	8	14		8	20	124	8	18		8	19	130	8	31	146
9	14		9	13½	116	9	21		9	17		9	17		9	31	
10	11½	110	10	14½		10	23		10	18	99	10	18		10	32	144
11	13½		11	16	112	11	22	120	11	16		11	17		11	32	
12	14½		12	16½		12	22	120	12	17		12	18	132	12	32	144
13	13½	108	13	18		13	23		13	16	99	13	18		13	33	
14	15		14	18		14	24	122	14	18	99	14	18	130	14	31	
15	11½		15	17	116	15	23	120	15	16		15	17		15	29	
16	13		16	16½		16	21	122	16	15		16	17		16	26	144
17	13½	104	17	16		17	23		17	15	99	17	16	132	17	25	
18	15½		18	17	116	18	23		18	16		18	15		18	24	
19	16		19	17		19	24	120	19	16		19	15		19	24	144
20	14½	106	20	16½		20	24		20	16	105	20	15		20	25	
21	14		21	17		21	24		21	14		21	15	124	21	25	
22	14½		22	17½	114	22	23		22	15	102	22	16		22	24	140
23	14		23	17		23	23	126	23	16		23	15		23	23	
24	14		24	17½		24	25		24	15		24	15	126	24	23	
25	15	104	25	18	116	25	24	126	25	15	102	25	14		25	24	144
26	14½		26	17½		26	24		26	16		26	13	126	26	22	
27	14	104	27	18		27	23	128	27	15		27	13		27	23	144
28	15		28	17½	118	28	24		28	16	104	28	13	123	28	21	
29	13½	104	29	18		29	25	128	29	15		29	13		29	21	144
30	14		30	18		30	24	128	30	16	102	30	13	120	30	21	144
1	10½	92	1	13	102	1	18	96	1	11	93	1	12	114	1	16	130
2	11		2	12½	96	2	16	96	2	10½	90	2	10	108	2	15	126
3	10½	92	3	14	94	3	14½		3	13	87	3	10	105	3	16	117
4	10		4	15	96	4	14½	92	4	11	90	4	10	105	4	15	114
5	9	90	5	15	94	5	15½		5	13		5	10	102	5	14	114
6	9½		6	15	94	6	14		6	12	84	6	11	99	6	13	110
7	9½	88	7	15	94	7	14	90	7	11		7	10		7	14	102
8	10½		8	14½	94	8	13		8	12		8	11	102	8	14	105
9	10	88	9	14½	92	9	14	90	9	9	84	9	11		9	14	104
10	11		10	14	94	10	13½		10	8		10	10	99	10	13	102
11	10		11	14	94	11	14	87	11	10	84	11	11		11	12	108
12	10½		12	14	92	12	13	85	12	9		12	11	102	12	13	102
13	10	88	13	14½	92	13	14	86	13	10	84	13	12	100	13	12	100
14	11½		14	14	90	14	14		14	11	81	14	11		14	12	102
15	11½	88	15	14	90	15	15	85	15	9	81	15	11	100	15	12	102

viously advanced by one of us (White, 1924b) that the diastolic tone of the cardiac musculature (the resistance to filling) is variable and diminishes after a few minutes of exercise. According to the best evidence the stroke volume of the heart is increased on exercise and this increase persists throughout the exercise. An increased extent of cardiac filling during exercise, when the period of filling is actually less than during rest, obviously means an increased rate of filling. If the filling of the heart is a passive process the rate of filling should be a function of the filling pressure alone. But work reported in the above paper showed that the venous pressure may return partially or completely to its resting level during the progress of the work at a time when the increased stroke volume of the heart is presumably being maintained. The conclusion is that the heart's resistance to filling has diminished.

The data of the three experiments on subject M. afford evidence of such a process. With light, moderate and heavy work there is an immediate rise of venous pressure, followed in a few minutes by a fall which is obviously not due to any further increase in heart rate. The data on subject D. are more difficult of interpretation. His venous pressure response to light exercise resembles qualitatively that of M. On moderate exercise there is an indication of a fall at from 8 to 10 minutes which, however, is succeeded by an increased level persisting to the end of exercise. On heavy exercise there is a progressive rise for about 10 minutes, the curve subsequently remaining almost flat with a slight upward trend, until the end of exercise. This maintenance of an increased venous pressure may mean that D.'s heart does not undergo the same extent of loss of tone as does M's. It may, however, merely mean that D. was working nearer to the limit of his reserve power than was M.

The question may be raised as to how far pressure determinations made on the veins of the back of the hand are indicative of the filling pressure of the right auricle. Pressure in the superior vena cava near the right auricle is, in the anesthetized dog, below atmospheric. Probably the most reliable figures are those of Burton-Opitz (1903) who gives 3 mm. Hg below atmospheric as the average pressure. From readings made at various points in the venous system he finds an average decrease of 1 mm. Hg for every 35 mm. length of vein. This gradient is of course steeper than in the human subject. If one considers the gradient of pressure in the region from venules to heart and assumes that this gradient is a straight line, that the vein wall maintains a constant tone, and that the venule pressure remains constant it is apparent that variations in the pressure in the vena cava will result in smaller variations in the pressure at a given point in a peripheral vein, but that the variations at the point measured will maintain a constant proportion to the variations in the vena cava, the value of this constant being determined by the location of the point of

measurement. Thus if the pressure at the arbitrary line where the venules form a vein is 17 mm. Hg, the pressure in the vena cava near the right auricle is 3 mm. Hg below atmospheric and the pressure at the point of reading is 7 mm. Hg then, from a consideration of similar triangles, it will be seen that a rise of 2 mm. Hg in the vena cava will result in a rise of 1 mm. Hg at the point measured. In other words, the variations of pressure in the vena cava are always greater than the variations in a vein on the back of the hand, assuming that the tone of the vein wall and the venule pressure, except as influenced by variations in the back pressure, remain constant.

It may be stated, however, that certainly the first and possibly the second of these assumptions is not warranted. Let us see how the failure of these assumptions to hold affects our case. In regard to the first, that the tone of the vein wall in a resting arm is unchanged on exercise of the lower extremities, it may be said that a conspicuous feature of the onset of exercise is a marked decrease in the calibre of the vein on the back of the hand in spite of the increased pressure of the vein. This decreased calibre is maintained throughout the exercise. The effect of the decrease of calibre which appears at the onset of exercise is to raise the pressure in the vein.

In regard to the second of the above assumptions, that the venule pressure remains constant except as influenced by variations in the back pressure, the evidence is not conclusive. Weber (1914) found an increase in volume of the resting forearm on exercise of the foot or of the opposite arm. Hewlett and van Zwaluwenburg (1909), however, find little or no increase in the blood flow through a resting arm immediately after exercise of the opposite arm. This latter finding has been corroborated many times by the students in the physiology course at this school, the usual result being in fact a decrease in blood flow. Since Weber's work points to a vasodilatation in the resting arm while the work with the Hewlett apparatus points to no change or a constriction, the question must be left open. In any event, as will be seen later, it is not of prime importance in our considerations.

We are now in a position to interpret the rise in venous pressure in the resting hand at the onset of exercise of the lower extremities. Our interpretation is that the rise is due to two factors, the venoconstriction and an impediment to the entrance of blood into the right auricle through the superior vena cava from the resting hand by the increased venous return from the active lower extremities. During the first few minutes of exercise the blood is returned to the heart at such a rate that the heart cannot receive it fast enough to keep the vena cava pressure down to its resting level. The question may now be raised as to what is the evidence that such an increase in the vena cava pressure occurs; cannot the increased tone of the wall of the vein in the resting hand be the sole explanation of

the rise in its internal pressure? The answer to this is that the venous pressure falls partially or completely to its resting level after a few minutes of exercise in spite of the persistence of diminished calibre of the vein.

There must then be some factor operating during the first few minutes of exercise to raise the venous pressure which diminishes or disappears as exercise proceeds and the only factor that could thus operate, since the venoconstriction persists, is an increased vena cava pressure. The fall in vena cava pressure which takes place after a few minutes of exercise must in turn be due to a diminished resistance to filling on the part of the heart. The only other possible factor which could produce this secondary fall of pressure in the resting hand vein would be a peripheral constriction in the hand coming on after a few minutes of exercise. There is no *a priori* reason or experimental evidence for believing that this occurs. Evidence that it does not occur is the fact that whenever a centripetal venous pulse is seen in the early stages of exercise it persists throughout the exercise. It is at least clear that if any increase in peripheral constriction of the resting hand does come on after a few minutes of exercise it is not sufficient to damp out the centripetal venous pulse. Furthermore, Hooker (1914) has shown that local changes in vascular tone affect the pressure in the peripheral veins little or not at all, the alterations in venous pressure being due principally to retrograde influences from the heart. This finding has been repeatedly confirmed (White, 1924a) (Eyster and Middleton, 1924). It is thus seen that variations in the venous pressure in the resting hand, while not an absolute measure of the variations in vena cava pressure, are proportional to them; also that the vena cava pressure variations are probably at least as great as the venous pressure variations.

The logical sequel of this argument is that during exercise the intrathoracic vena cava pressure may rise considerably above atmospheric. Additional evidence that this is the case is afforded by the following experiments on the response of jugular pressure to exercise. The subject lies on his back on a mattress with the head and shoulders supported so that the level of the external jugular vein at the point of pressure determinations is 2–3 cm. below the level of the anterior chest wall over the sternum. The capsule is applied as close to the thorax as possible, about 3 cm. above the clavicle. In this position the vein is seen to collapse on inspiration and to be visible during expiration. Two subjects were used; in each the resting jugular pressure was between 1 and 3 cm. water during expiration. The subject now exercised for 3 minutes by alternate flexion and extension of the lower extremities, breathing through the mouth to lessen the chances of performing the Valsalva experiment. With each subject the jugular pressure rose to 7 to 10 cm. water on exercise and a positive pressure was maintained during inspiration. With a pressure this much above atmospheric in the external jugular during exercise it is certain that the pressure in the thoracic venae cavae is above atmospheric.

SUMMARY

The response of the venous pressure, arterial pressure, pulse rate and respiratory rate to a 10-minute period of static exercise has been followed in a series of 8 experiments on 7 normal subjects. The exercise consisted in holding the lower extremities horizontal while sitting in a chair. The response of the venous pressure and pulse rate to a 30-minute period of dynamic exercise has been followed in a series of 6 experiments on 2 normal subjects. Each subject engaged in a period of light, of moderate and of heavy exercise on the bicycle ergometer.

The data are believed to warrant the conclusion that the tendency of the increased venous return during exercise to raise the venous pressure is partially or completely compensated by the venodilator mechanism, the increased aspiratory action of the thorax, the increased heart rate and a loss of diastolic heart tone, the first three factors coming into operation immediately at the onset of exercise, the fourth after a few minutes. On heavy exercise the compensatory process does not suffice to keep the venous pressure level constant. The extent to which the compensatory process operates in various normal subjects performing the same type and severity of exercise is quite variable. Since two of the factors, the pulse rate and aspiratory action of the thorax, are invariably increased on exercise and since venodilatation also is probably an invariable accompaniment of exercise it appears probable that the variability of the compensatory response is due principally to variation in the extent of diminution of diastolic heart tone.

The experiments on static exercise afford evidence that the pumping action of the skeletal muscles is not an essential factor in bringing about an increased venous return. The static exercise data seem to lend support to the view advanced by Danzer that there is a peripheral vascular dynamic mechanism which aids in the return of blood to the heart; the effect of this mechanism is increased on exercise.

Most normal resting subjects even while awake and with the hand at room temperature show when an appropriate pressure is applied a centripetal pulse in the veins of the back of the hand.

The pressure in the great veins of the thorax may rise above atmospheric during exercise.

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STUDIES ON RADIATED PROTEINS

I. Coagulation of Egg Albumin by Ultraviolet Light and Heat

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In an earlier paper the coagulation of egg albumin by ultraviolet light was studied in relation to the isoelectric point (1922). These early results have been extended and modified, using more carefully purified albumin, and the coagulation of egg albumin by ultraviolet light and heat has been found so similar in some respects, and so dissimilar in others, that the results lead to interesting speculation as to the mode of action of these two agents.

Method. Pure crystalline egg albumin was prepared by the modification of Hofmeister's method given by Taylor (1916). Equal volumes of egg white and saturated ammonium sulphate were beaten to a stiff froth and left to stand over night. The precipitate of globulin and mucoid was then filtered off and a 10 per cent solution of acetic acid added to the filtrate until a slight permanent precipitate was formed. Then 1 cc. of the 10 per cent acid was added for each 100 cc. This gave a bulky precipitate which became crystalline on standing over night. It was filtered and the crystals washed three times with half saturated ammonium sulphate, containing 0.1 per cent acetic acid. The crystals were then dissolved in the minimum of water and saturated ammonium sulphate added until a precipitate formed. Then 2 cc. more were added to each liter and the albumin recrystallized in 24 hours. This process was repeated five times to remove all colloid impurities. Finally, the crystals were dissolved in pure water and dialysed two days to remove the ammonium sulphate. After two days' dialysis, with frequent changes of water, the solution of colloidal egg albumin was free of salts and had a hydrogen ion concentration corresponding to pH 4.8. This is isoelectric egg albumin. In previous work (1922) when the albumin was not recrystallized to remove all impurities, the dialysed albumin had a pH of 6.2, showing the presence of impurities and the results were not wholly satisfactory. Prepared by the above method the albumin seemed pure and was always obtained in isoelectric condition.

In studying the coagulation of egg albumin by heat and radiation, at different hydrogen ion concentrations, no buffers were used since they

very materially modify the reaction of proteins to these two agents. To adjust solutions to the desired pH values a few drops of very dilute hydrochloric acid or sodium hydroxide were used. To get the reaction to heat the solutions were brought to 100°C. in a water bath. Ultraviolet radiation was given by means of a Hanovia quartz mercury arc at a distance of six inches. The solutions were exposed in quartz test tubes and were given an exposure of half an hour or more, depending on the degree of opalescence required. There was no coagulation through glass so only wave lengths shorter than 310 mμ were effective. The arc was run at 58 volts and 3 amperes, on 110 D. C., and gave 1 lithopone unit in 15 seconds at a distance of 6 inches. The pH was determined colorimetrically by means of the following indicators: phenol red (pH 6.8 to 8.4), brom cresol purple (pH 5.2 to 6.8), brom cresol green (pH 4.0 to 5.6), brom phenol blue (pH 3.0 to 4.6).

RESULTS. *Coagulation with ultraviolet light and heat:* The results obtained by means of heat and radiation were exactly similar at different pH values, insofar as visible coagulation was concerned. This striking similarity between heat coagulation and light coagulation has not, I believe, been noted before, and the results are given in detail in table 1.

It is evident from table 1 that salt-free egg albumin will precipitate in flakes at the isoelectric point if radiated with ultraviolet light or brought to 100°C. For a narrow region on the acid side, and a longer region on the alkaline side of the isoelectric point, heat and radiation produce an opalescence which will not filter clear. However, on bringing the opalescent solutions to the isoelectric point they precipitate in fine flakes and filter clear. The opalescence on radiation is much less dense than that produced by heating. This is due to the fact that all the molecules are affected by heat while, owing to the absorption of ultraviolet light by the albumin solution, only the molecules nearest the light are affected. Convection currents eventually bring all the molecules into a position to be affected by the radiation but it takes a long time (2 to 3 hours) to remove all the protein from solution. This has been done, however, at all values of pH between 4.0 and 7.0 and probably can be done over a much wider range still. When radiated at any pH, except 4.7 to 4.9, the solution after radiation was brought to pH 4.8, filtered clear, and the filtrate boiled to test for the presence of any protein left uncoagulated by light.

At hydrogen ion concentrations less than pH 4.4 or greater than pH 5.6, where the albumin molecules have to some extent reacted with the acid or alkali to form protein chloride or sodium proteinate, with subsequent dissociation into positive and negative protein ions, the solutions remain perfectly clear after radiation. The protein molecules have, however, undergone a transformation since they precipitate when brought to pH 4.8 either by the addition of acid or alkali or by dialysis against

TABLE 1

Results on treating dialysed egg albumin with heat (100°C.) or with ultraviolet light

pH 4.4 OR LESS	pH 4.5	pH 4.6	pH 4.7, 4.8, 4.9	pH 5.0 – 5.3	pH 5.4, 5.5	pH 5.6 AND HIGHER
Clear but will precipitate in fine flakes on bringing to 4.8	Faint opalescence which precipitates on bringing to 4.8	Heavy opalescence → flakes at 4.8	Precipitates in flakes which are fine in case of radiation and large in case of heat. Filters clear	Heavy opalescence at 5.0. Degree of opalescence decreases with increasing pH. All → flakes at 4.8	Faint opalescence which → flakes on bringing to 4.8	Clear but → opalescence if brought to pH 5.5 – 5.0 and → flakes at pH 4.8

distilled water. They not only precipitate on dialysis but also on one-half saturation with ammonium sulphate, after radiation or heating at any pH. They seem, in fact, to have been transformed into globulins, a fact noted by Schanz (1915), but it is probably more accurate to say that radiated and heated albumins react like globulins, as they may be in a globulin-like condition without having been actually transformed into globulins. These radiated albumins also have a very characteristic odor which is exactly similar to the odor of heated albumin solutions.

The opalescence formed by heat and light is partially reversible. If the solutions are heated or radiated at a pH greater than 5.6, or less than 4.4, where no visible opalescence is formed, then the opalescence and precipitate formed by bringing them to the isoelectric point clears on bringing them to pH 4.2 or 5.8 with more acid or alkali. If, however, the solutions are radiated between pH 4.6 and 5.5, where opalescence or precipitation forms during radiation, the opalescence is only partially reversible. If very weak alkali is added to an opalescent solution radiated or heated at pH 5.2 it will clear slowly, still showing a faint opalescence at pH 8.4, which, however, clears on standing. If acid is added while there is still a moderate opalescence (pH 6.8 for instance) the original degree of opalescence is obtained at pH 5.2. But if it is brought to pH 8.4 and allowed to stand until clear, a much smaller degree of opalescence is formed on bringing the solution back to pH 5.2. If acid is added to a solution precipitated by light or heat at the isoelectric point there is very little clearing. The flakes can be redissolved by bringing the solutions to pH 4.0, and shaking vigorously, but there is a heavy opalescence which diminishes very slowly with increasing acidity, being still marked at pH 3.0.

It is evident from table 1 that the end result of the absorption of light or heat energy by the colloidal particles of egg albumin is the same. Both agents bring about some molecular rearrangement in the colloid resulting in coagulation when it is brought near its isoelectric point, either before or after radiation or heating. It is, however, probable that this rearrangement is initiated in a somewhat different way by these two agents. This is probable from theoretical considerations. The resonator that is set in motion in an atom or molecule when ultraviolet light is absorbed is known to be one of its constituent electrons. This activated electron may be ejected from the molecule, as the result of absorption of light energy, in which case the molecule is ionized, or it may be displaced to an outer orbit, in which case the atom or molecule is activated. In either case it will show altered chemical reactions. The resonator when heat is absorbed is probably an atom or group of atoms in the molecule. It is also possible to have electrons displaced to outer orbits by the absorption of heat but they would not be ejected from the atom except at very high temperatures (Richardson, 1921), in the neighborhood of 400°C.

There is also experimental evidence for believing that, although coagulation of proteins by light and heat is so similar in its end result, the mechanism involved is different. It is possible under suitable conditions to desensitize egg albumin to heat coagulation by previous radiation with ultraviolet light, and this certainly suggests that the two coagulating agents act in a different way.

Desensitization to heat coagulation: In a preliminary communication (Clark, 1925), it was stated that "solutions heated between pH 6.2 and 8.0 showed a faint opalescence while solutions radiated at pH greater than 6.2 remained clear but were desensitized to heat." It was found out later that this faint opalescence produced by heating at pH 5.6 to 8.0 was due to the presence of a colloid impurity which is probably non-crystallisable egg albumin, called conalbumin by Sörensen. When the albumin is purified as described above by repeated crystallizations, no opalescence is produced either by heat or light at pH 5.6 or higher. If, however, the washing is taken from the first crystallization, precipitated by adding ammonium sulphate, filtered and dialysed, then the dialysate has a pH of 4.8 and is presumably a solution containing a considerable amount of non-crystallisable egg albumin. When this solution is brought to different pH values and heated to 100°C., it is clear at pH 4.0, coagulates in flakes at 4.8, shows heavy opalescence at 5.8, medium opalescence at 6.2, and faint opalescence at 7.0. When it is radiated with ultraviolet light it is clear at 4.0, precipitates in flakes with some opalescence at 4.8, shows medium opalescence at 5.8 and is clear at 6.2 and 7.0. When radiated at pH 6.2 and 7.0 the solution is desensitized to heat coagulation at these values of hydrogen ion concentration.

Therefore some impurity, which is not a globulin as it does not precipitate on dialysis, and which is probably conalbumin, is capable at pH 6.2 to 7.0 of giving a faint opalescence on heating but not on radiation and can be desensitized to heat coagulation at this pH by radiation. Pure crystalline egg albumin, however, shows no coagulation with either light or heat at pH values above 5.6, and no desensitization to heat when radiated at these pH values, as is shown by the results given in table 2.

However, desensitization to heat is possible with pure crystalline egg albumin under very special circumstances. When the egg albumin is radiated at 0°C. (i.e., with the quartz tubes packed in crushed ice) it remains perfectly clear at all values of hydrogen ion concentration except pH 4.8, which shows a very faint opalescence. This fact has been noted by Bovie (1913). The tube radiated at pH 4.8 is not desensitized to heat as it coagulates in heavy flakes on heating but a tube radiated one hour at 6 inches from the arc, at 0°C. and pH 5.0, shows a slight desensitization and those radiated at pH 5.2, 5.3 and 5.4 show a considerable degree of desensitization to heat. A tube at pH 5.4, radiated two hours at 0°C.,

was perfectly clear but on standing showed a barely perceptible trace of opalescence which did not increase on boiling, so that the solution was totally desensitized to heat coagulation. These results are summarized in table 3.

The solution which was radiated for two hours at pH 5.4, and 0°C., and was completely desensitized to heat, showed complete precipitation on one-half saturation with ammonium sulphate. It is evident, therefore, that all the molecules of egg albumin had undergone a transformation as the result of the absorption of light energy and had been put into a globulin-like condition in respect to precipitation by salts.

TABLE 2

Effect of radiation of egg albumin (pH 6.0) on subsequent heating

	TREATMENT	RESULT
a	Radiated at 30°C., brought to pH 4.8	Opalesence
b	Radiated at 30°C., heated, brought to pH 4.8	Heavy opalescence
c	Radiated at 0°C., heated, brought to pH 4.8	Heavy opalescence
d	Radiated at 30°C., brought to pH 4.8 heated	Heavy opalescence
e	Radiated at 0°C., brought to pH 4.8 heated	Heavy opalescence
f	Untreated, heated brought to pH 4.8	Heavy opalescence

TABLE 3

Desensitization to heat after radiation at 0°C.

pH	RADIATED 1 HOUR AT 0°C.	HEATED TO 100°C. AFTER 1 HOUR RADIATION	HEATED AFTER 2 HOURS RADIATION
4.8	Faint opalescence	Heavy precipitate, no desensitization	
5.0	Clear	Medium opalescence, some desensitization	
5.2	Clear		
5.3	Clear	Faint opalescence, marked desensitization	Complete desensitization
5.4	Clear		

This desensitization to heat coagulation by radiation makes it seem probable, on experimental grounds, that light and heat attack the albumin molecule in different ways. The result is that if the albumin is radiated under conditions, such as low temperature, where light coagulation is prevented, the radiated molecule is no longer able to react to heat. It has been noted by Howell (1921) that fibrinogen after radiation with ultraviolet light, or with visible light in the presence of a sensitizer, will neither coagulate on heating, nor on the addition of thrombin, so that the desensitization described above is not an isolated phenomenon.

MODE OF ACTION OF LIGHT AND HEAT. *Hydration and dehydration theories*: The above stated facts should give a clue to the mode of action

of light and heat in the coagulation of proteins. Chick and Martin (1910) found that the heat coagulation of egg albumin follows the law of monomolecular reactions, the coagulation rate being at any moment proportional to the concentration of protein. They came to the conclusion from their experiments that heat coagulation consisted of two processes, the first a hydration, or union between protein and hot water, which they called denaturation, the second a subsequent agglutination of the denatured protein particles. According to this scheme the results given in this paper might be interpreted by saying that hydration, or denaturation, of the egg albumin molecules can proceed at any pH but that, in salt free solutions, the agglutination of these hydrated molecules would only occur if the solutions are brought to a pH between 4.5 and 5.5, either before or after heating.

It is not, however, certain that the initial reaction is a hydration. It has been thought by a number of investigators, notably Pauli and Robertson, that the coagulation of proteins is essentially a dehydration. Robertson (1918) points out that the monomolecular reaction formula would apply either to the hydration or the dehydration of the protein molecule. Pauli (1922) and his pupils have found the influence of a variety of salts on the coagulation temperature of proteins, in acid and alkaline solutions, to be in agreement with the view that heat coagulation like salt and alcohol coagulation is accomplished through the dehydration of protein molecules.

Pauli states that all colloids which are stable in neutral solution are hydrated, while those which are unstable at the isoelectric point are hydrophobe or anhydro-colloids. Untreated egg albumin belongs to the first class and egg globulin and heated egg albumin to the second class. Pauli believes that anhydro-colloids such as globulins form heavily hydrated particles in solution when electrically charged, this being presumably due to an electrical adsorption of water molecules at the surface of the charged protein particles. These colloids precipitate in the electrically neutral state as the particles lose their water of hydration when discharged. Albumins, on the other hand, he believes, still hold some water molecules in an electrically neutral state and are thereby kept from spontaneous precipitation when brought to the isoelectric point. These residual water molecules must be an integral part of the protein particle and not due to electrical adsorption.

Now egg albumin, when radiated or heated at the isoelectric point, forms a precipitate like a globulin brought to this point, and the similarity between radiated or heated albumins and globulins is even more striking if the albumin is radiated or heated at a pH less than 4.4 or greater than 5.6. It seems reasonable to suppose, therefore, that radiated or heated egg albumin is an anhydro-colloid, similar to egg globulin, if not identical with it, and this view was given in a preliminary communication (Clark, 1925).

However, in order to test the feasibility of this hypothesis, a few experiments were made on the viscosity of radiated and heated albumin, which indicate that, although dehydration may be involved in the second step of coagulation, the first step, called denaturation by Chick and Martin, is probably a hydration, as originally supposed by them.

Viscosity: An Ostwald viscosity tube was used with a very fine capillary so that the time of flow of 2 cc. of distilled water was approximately eight minutes at room temperature. A solution of egg albumin was used of the density obtained after dialysis with the method given above. From the values obtained for the viscosity this would seem to be about a 2 per cent solution. In the work on heat and light coagulation this solution was diluted to twice the volume with distilled water. Measurements were made only at pH 6.0, the object of the investigation being to determine whether egg albumin, "denatured" by heat or light at this pH, where no

TABLE 4

Viscosity of radiated and heated egg albumin (pH 6.0)

MATERIAL	TIME OF FLOW THROUGH CAPILLARY				COEFFICIENT OF VISCOSITY ($H_2O = 1$)	
	18°C.		25.5°C.		18°C.	25.5°C.
	min.	*sec.*	*min.*	*sec.*		
Water	9	24	8	0		
Untreated albumin	9	54	8	24	1.05	1.05
Radiated albumin	9	53	8	27	1.05	1.05
Heated albumin 6.4	11	14	9	4	1.20	1.21
Heated albumin 6.0	11	14				

visible coagulation takes place, showed any evidence of dehydration or not. On heating there is a change in hydrogen ion concentration to pH 6.4 so that, with the heated sample, viscosity measurements were made, both at pH 6.4, and after readjustment to pH 6.0. The results are given in table 4.

These results show that there is absolutely no change in the viscosity of egg albumin when radiated at pH 6.0, where no coagulation takes place, but where the albumin has been modified or denatured so as to behave like a globulin. According to Einstein's formula, viscosity is a linear function of the relative volume occupied by the solute in the solution: $\eta = \eta_o (1 + k \phi)$ where η is the viscosity of the colloidal solution, η_o that of the water at the same temperature, k is a constant, and ϕ is the fraction of the volume occupied by the solute in the volume of the solution. This formula holds well for solutions of low viscosity. It therefore seems reasonable to suppose that no hydration or dehydration has taken place in the egg albumin as the result of radiation at pH 6.0. A change in the aggregation may have taken place, however, as this would not affect

the viscosity provided there was no change in the distribution of water between the two phases.

From Einstein's formula a rise in viscosity means that water has been taken into the dispersed phase so that the results with heated albumin indicate hydration of the egg albumin after heating. Ostwald (see W. M. Bayliss, *Principles of General Physiology*, p. 243, 1915) has shown a remarkable change in the viscosity of egg albumin during the process of heat coagulation in egg white. There is first a fall in viscosity and then a sharp rise at 57° just before the appearance of turbidity. This is followed by a sharp decrease, and then a steady fall as coagulation proceeds. This, in conjunction with the results given above, suggests the following picture of the process of heat coagulation. The first part of the process, called "denaturation" by Chick and Martin, which is obtained alone when the

TABLE 5

Surface tension of egg albumin

SUBSTANCE	pH	SURFACE TENSION	SURFACE TENSION AFTER RADIATION	SURFACE TENSION AFTER HEATING (100°)
		dynes per cm.	*dynes per cm.*	*dynes per cm.*
Water		76		
Albumin	8.4	74		
	7.0	75	61 (1 hr. rad.)	72
	6.0	71	66.5 (½ hr. rad.)	70.5
	5.4	67		
	5.2	67		
	4.8	68	64 (coag.)	
	4.0	69	62 (1 hr. rad.)	69
	3.0	68		

albumin is heated at a pH greater than 5.6 or less than 4.4, is accompanied by hydration as Chick and Martin supposed. This view is borne out by the rise in viscosity in heated albumins at pH 6.0 (table 4) and by the steep rise in Ostwald's curve just before coagulation takes place. The second part of the process, the agglutination of the denatured particles, may be accomplished by dehydration as assumed by Robertson and Pauli although there is no proof of this at present. This would account for the sharp decrease in Ostwald's curve after coagulation sets in. These changes in degree of hydration are undoubtedly accompanied by changes in structural formation to which at present we have no clue.

Surface tension: As it seemed probable that the change produced by heat and radiation might bring about interesting changes in surface tension, some preliminary measurements were made with a duNoüy tensiometer. The same strength albumin solution was used as in the viscosity determination, and the results were so promising that further experiments on the time drop of surface tension in radiated solutions, at different

values of hydrogen ion concentration, are contemplated. The results are given in table 5.

In these experiments only the initial surface tension was measured but the results were very concordant when several measurements were made in rapid succession. On standing over night in the ice box, the surface tensions of the radiated and heated albumins did not change at all. Buglia (1908) and Frei have shown that the surface tension of gelatin solutions and blood serum is increased by the addition of small quantities of alkali and diminished by the addition of small quantities of acid. This is similar to the change in surface tension with pH in table 5. With the preliminary results given above only a suggestion of their meaning can be given but the very marked drop in the surface tension after radiation is extremely suggestive.

J. J. Thomson (1923) has given us the conception of what he calls a polar molecule, that is one in which the electrical charges are not symmetrically placed, and which consequently has a finite electrical moment. This conception has led to very satisfactory results in calculating the dielectric constant of liquids. Among the groups that show a definite polarity are the OH and COOH groups. Langmuir (1917) has shown that in oleic acid it is the COOH group that is attracted by the water molecules so that the oleic acid molecules are polarized on the surface of the water with the COOH groups in contact with the water and the CH_3 groups sticking out. The number of polar groups in a molecule will therefore affect the orientation of the molecules in the surface film, the area occupied by the molecules and consequently their power of lowering surface tension.

It seems possible that the change in surface tension with pH may be associated with the ionization of the COOH group in an alkaline medium, and a consequent reorientation of the surface molecules. On the other hand, it may be due to the fact that the electrical double layer formed at the air-water interface, when the latter contains an unbalanced number of positive and negative ions, is such that neutral and positively charged egg albumin particles lower the surface tension more than negatively charged albumin.

If this idea proves tenable the fact that radiated albumin lowers the surface tension so markedly supports the theory, previously advanced by the author, that ultraviolet light acts by freeing electrons. This would diminish the negative charge and tend to give radiated albumin the low surface tension characteristic of positively charged albumin. Since sufficient radiation gives egg albumin a surface tension lower than that of positively charged albumin at pH 4.0, it also seems possible that the molecule may have been broken down, under the action of light, giving smaller aggregates.

The action of heat is to decrease the surface tension a little but only

when it is high to begin with. This slight effect can hardly indicate any change in charge or state of aggregation.

Electrophoresis: A large number of experiments were performed to test the theory that molecules lose electrons on absorbing ultraviolet light. This should of course modify their motion in an electric field. The experiments were not very satisfactory owing to the necessity of omitting buffers and the consequent change in pH near the electrodes. The experiments were on the whole confirmatory of the theory that ultraviolet light ionizes the protein molecule, and will be published in a subsequent paper after further confirmation.

CONCLUSION

Salt-free egg albumin is so modified by the absorption of ultraviolet light, shorter than 320 mμ, or heat (100°C.) that it coagulates when radiated or heated at certain values of pH near the isoelectric point, the range of coagulation and the nature of the coagulation being identical for both agents.

When radiated or heated at any other pH the albumin remains clear but reacts like a globulin, forming a precipitate on half saturation with ammonium sulphate, or on dialysis.

The initial action of light and heat is however different because; 1, it is possible under very special conditions of pH and temperature to desensitize egg albumin to heat by radiation; 2, albumin heated at pH 6.0 shows a marked rise in viscosity while albumin radiated at this pH shows no change in viscosity; 3, albumin radiated at pH 6.0 shows a marked drop in surface tension while albumin heated to 100°C. at this pH shows practically no change in surface tension.

The change taking place at pH 6.0 on heating or radiating is probably the first part of the coagulation process, or denaturation, the second part of the process, which consists in the agglutination of the denatured particles, taking place only near the isoelectric point in salt-free albumin. The changes in viscosity and surface tension indicate that denaturation by radiation does not involve any change in the water content of the disperse phase but is probably associated either with a change in charge, due to loss of electrons on radiation, or with a change in the state of aggregation, or both. On the other hand, the viscosity and surface tension results indicate that the denaturation by heat is accompanied by hydration of the albumin particles with no change in the state of aggregation. The second part of the process, the agglutination of the denatured particles, appears to be the same with either light or heat, as it takes place under exactly similar conditions. There is no evidence in this work to suggest the nature of this second process but it is possible that it may involve dehydration.

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THE ACTION OF ADRENALIN ON RESPIRATION[1]

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Several investigators have observed that the introduction of adrenalin into the blood stream of experimental animals influences the rate and depth of respiration. Oliver and Shäfer (1) found that extracts of adrenal glands produce shallowness in the depth of respiration. Boruttau (2) obtained a similar effect on respiration and believed that it was due to the effect of adrenalin on the respiratory center. Langley's (3) results agreed with those of Oliver and Shäfer; he also observed that succeeding doses brought forth insignificant responses. Langlois and Gerrelon (4) showed that either a diminution in depth or an increase in the rhythm of respiration might occur in the dog when adrenalin was introduced and concluded that the action depended on the condition of the respiratory center. Nice, Rock and Courtright (5) have shown that small doses of adrenalin in general cause an increase in the depth of respiration while large doses produce shallowness in the dog and cat. These results were attributed to the action of adrenalin on the respiratory center. Jackson (6) has also obtained an increase in the depth of respiration after injecting adrenalin. Boukaert (7) found a diminution of respiration in the cat after ergotoxin, which changes the adrenalin effect on blood pressure into a depressor action (8) and concluded that the result depends on the condition of the respiratory center at the time. Thompson, Sturgis and Wearn (9) found an increase in respiration after the injection of adrenalin in men and concluded that this is due to increased metabolism. Boothby and Sandiford (10) obtained similar results.

Roberts (11) has brought forth evidence which has led him to the conclusion that the influence of adrenalin on respiration is dependent on its effects on the circulatory system. In fact he thinks the adrenalin action is due to anemia of the respiratory center. He also attributed the Cheyne-Stokes respiration which occurred in his animals to adrenalin. Hugett and Mellenby (12) supported Roberts in their first paper but their later work (13) gave contrary results, and they think that adrenalin affects the respiratory center directly, supporting the opinion of former workers (2), (4), (5), (7).

[1] A preliminary report of this investigation was presented at the meeting of the American Physiological Society in St. Louis, December, 1923.

In this paper we have studied the relation of the respiratory changes to the blood pressure effects in the head and body areas, after administering adrenalin, with the hope of confirming or disproving the anemic theory.

Methods. Cats, dogs and rabbits were used in our experiments; most of the observations were made under urethane anesthesia but a few were done on decerebrate cats. The respiratory movements were recorded from the diaphragm as in our previous experiments (5). This method has the advantage of not interfering with the free interchange of air to and from

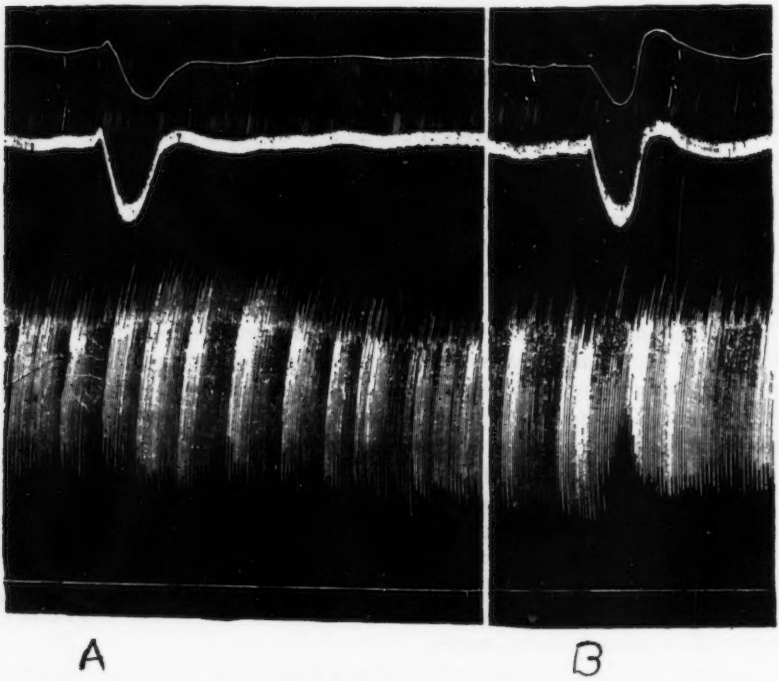

Fig. 1. Dog. At A, 0.5 cc. of 1:100,000 adrenalin was introduced. At B, 2 cc. of 1:100,000 adrenalin solution was given.

the lungs. A tambour attached to a Y tracheal cannula was found to be too insensitive to record small changes in respiration. The blood pressure was synchronously recorded by two mercury manometers, one from the central end of a carotid and the other from the peripheral end of the same artery, the carotid being ligated beyond the branch of the internal carotid in most of the experiments.

The adrenalin used was that of Parke, Davis & Company. Various strengths from 1:200,000 to 1:100,000, the dilutions being made with

normal saline, were introduced either into the central end of a jugular vein or into the peripheral end of a carotid artery.

Results. In figure 1 two effects on respiration are seen when different doses of adrenalin were introduced. In A, there is an increase in the depth of respiration following the introduction of 0.5 cc. of 1:100,000 adrenalin into a small dog. This strength of adrenalin caused a fall of blood pressure in the central and peripheral ends of the carotid artery. In B, the dosage was increased to 2 cc. of 1:100,000 adrenalin. In this case the blood pres-

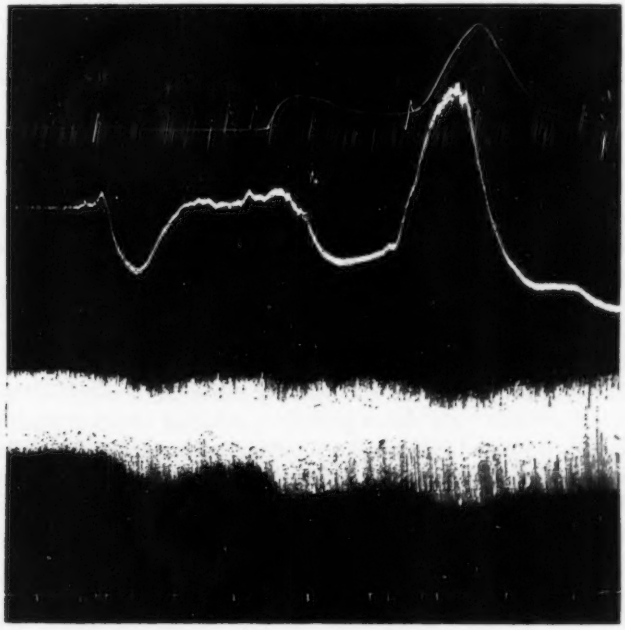

Fig. 2. Cat urethanized. At A, 0.5 cc. of 1:200,000 adrenalin solution was introduced; at B ½ cc. of 1:200,000 and at C, 3 cc. of 1:200,000 was introduced.

sure is lowered in the central and peripheral ends of the carotid as in A, but there is a reversal, or decrease in the depth of respiration.

In figure 2, when 0.5 cc. of 1:200,000 adrenalin was introduced into a cat there is a fall in the blood pressure in the central end of the carotid, also a slight fall in the peripheral end of the same artery but a considerable increase in the depth of respiration; at B, 1.5 cc. of 1:200,000 was introduced and a fall in the blood pressure in the central end of the carotid occurred, but a rise in the peripheral end of this artery with a decided increase in the depth of respiration, when shallowness should have occurred

under the anemic theory. At C, the dosage of adrenalin was increased to 3 cc. of 1:200,000; there is a rise in the blood pressure in both the central and peripheral ends of the carotid artery but still an increase in the depth of respiration. These curves show that there is a range in which there is no definite relation between changes in blood pressure in the head area (circle of Willis) and the effects on respiration.

Discussion. In regard to the anemic theory, our results demonstrate that within limits there may be an increase or a decrease in the depth of respiration after the introduction of adrenalin into the blood stream and this may be independent of the effects on blood pressure. In fact we have obtained a decrease or an increase in the depth of respiration along with a fall or a rise in the blood pressure in the head area, and an increase or a decrease in the depth of respiration accompanied by a rise and a fall in blood pressure in both the head and body areas. Large doses of adrenalin produce apnea and invariably cause a rise in blood pressure, as is well known.

In rabbits where adrenalin produces only a constrictor effect on blood pressure (14) we have obtained increases in the depth of respiration after the administration of small doses.

SUMMARY

1. Within limits small doses of adrenalin injected into the blood stream of anesthetized cats, dogs and rabbits may cause an increase in the depth of respiration, while large doses produce a reversal effect or shallowness.

2. There is a range in which the effects of adrenalin on respiration are independent of its influence on blood pressure. Large doses, however, invariably produce apnea and a rise in blood pressure.

3. Our results do not support the anemic theory.

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HEAT REGULATION AND WATER EXCHANGE

IX. The Mechanism of Cold Anhydremia and Edema[1]

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We (1925) have recently demonstrated that water lost from the blood in the response of the body to environmental cold is transferred to the skin, subcutaneous tissues and muscles of the cooled regions. This finding suggests the extension of the theory of heat regulation in such a way as to account, at least tentatively, for the various steps by which a cold stimulus brings about this movement of fluid. The investigations of Krogh (1922) have indicated that if the colloidal pressure of the serum is normal the amount of fluid leaving the capillaries increases only if their walls become more permeable. Increased capillary permeability is closely associated with capillary dilatation, which has been observed to be a constant sequel to anoxemia.

In the response to cold the anoxemia is undoubtedly the result of the constriction of the arterioles, as is shown by the fact that the skin first turns pale, upon the application of cold, then purplish red and then blue. That the oxygen saturation is reduced under these conditions has been shown by Meakins and Davies (1920). Thus we have an explanation of how cold gives rise to the edema described in the first paper referred to above.

The question next arises as to the mechanism of the vaso-constriction. That neither a hormone nor a central mechanism can be held fully responsible is shown by the unilateral distribution of the edema. Still further doubt is cast upon the possible rôle of hormones by certain facts relating to the adrenal and pituitary glands.

The influence of the adrenals as an essential factor is excluded by the fact that splanchnotomized dogs respond to cold baths by concentration (fig. 1). Furthermore, Hartman's (1923) experiments have indicated that epinephrin is not produced as a result of the cold stimulus in sufficient quantities to account for the concentration.

The possibility of pituitary influence seems to be excluded by the fact that pituitary extracts tend to tighten capillaries rather than increase

[1] These investigations have been assisted by a grant from the Ella Sachs Plotz Foundation.

their permeability (Krogh, 1922), and there is little evidence that suc extracts concentrate the blood significantly. In Lamson's (1923) experiments pituitary extract not only produced no anhydremia but tended to prevent such an effect from epinephrin. While Kolls and Geiling

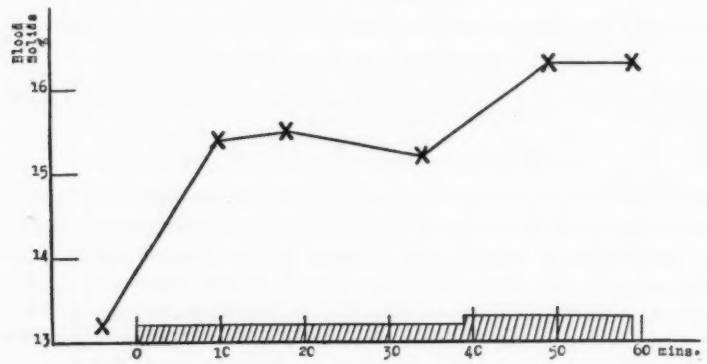

Fig. 1. Splanchnotomized dog: effect on blood solids of cold "hip bath." Upper part of body exposed to warm chamber to prevent shivering. After first 35 minutes dog was immersed in same bath up to the neck. No increase in muscular tone could be detected in the "hip bath."

(1924) in three experiments on unanesthetized dogs observed some fleeting anhydremia resulting from pituitary injections, the literature which they quote is contradictory. We have examined this point in two dogs and in both found hydremia. This was preceded in one animal by a slight blood concentration (excitement ?) as shown by the following:

TIME	SPECIFIC GRAVITY	TIME	SPECIFIC GRAVITY
2:49	1.0458 (average of three determinations)	3:38	Injection 2 cc. 0.9 per cent NaCl intravenously
3:05	Injection of 1 cc. pituitary liquid intravenously	3:40	1.0457
		3:43	1.0458
3:06	Panting	3:48	Injection 2 cc. pituitary liquid intravenously
3:06–3:34	1.0487		
	1.0453	3:50	1.0471
(Approximately	1.0471	3:51½	1.0471
two minute	1.0473	3:52½	1.0458
intervals)	1.0452	3:55	1.0468
	1.0457		
	1.0463		
	1.0453		
	1.0443		
	1.0445		
	1.0465		
	1 0452		

The hydremia was particularly well exhibited by the other dog. After an hour the blood concentration began to show a reversion toward normal as seen both from specific gravity and solids determinations:

TIME	SPECIFIC GRAVITY	SOLIDS	TIME	SPECIFIC GRAVITY	SOLIDS
4:11½	1	19.25	4:43	1.0464	
		19.15	4:45	1.0480	
4:12	1.0512		4:47		17.86
4:14	1.0511				17.92
4:16	1.0517		4:47	1.0486	
4:17	1.0516		4:52	1.0480	
4:24	Injection 2 cc. pituitary liquid intravenously		4:52½	1.0490	
			4:57	1.0481	
4:24½	Panting				
	1.0512		5:21	1.0480	
4:26	1.0522		5:21½	1.0497	
4:27	1.0513		5:22½	1.0479	
4:27½	1.0519		5:23½	1.0497	
4:28	1.0516		5:24½	1.0493	
4:37	1.0494		5:25½	1.0493	
4:40½	1.0485		5:26		18.38
					18.35

These two experiments confirm Krogh's idea that pituitary tightens the capillaries, and show that it can not account for cold anhydremia.

That the central nervous system is not essential to the fluid shifting produced by cold is seen in the following experiment. A dog whose lumbar sympathetic chain had been previously removed under morphine and ether was given a cold hip bath in a warm room:

TIME	SPECIFIC GRAVITY
2:45	1.0356
2:49	1.0350
3:02	In hip bath 14.5°C., in warm room 35.5° C., wet kata, 7.2 millicalories
3:05	Shivering
3:18½	1.0376
3:19	1.0387
3:23½	1.0376
3:25	Out of bath, remains in warm room
4:34	1.0357
4:36½	1.0343

The blood concentrated and there must have occurred a corresponding edema.

Since the evidence from the unilaterally cooled dogs (Hamilton and

Barbour, 1925) indicates that water leaves the blood in the cooled areas only, one must assume that in a hip bath experiment the edema will occur only in the hind limbs. As the vaso-constrictor paths to this part of the body had been entirely eliminated by the operation, the fluid transfer could not have been brought about by reflex vaso-constriction. Of course an inhibition of the dorsal root vaso-dilators might still be considered a remote possibility.

That shivering is unrelated to cold anhydremia and edema we have shown by two experiments similar to the one just cited, with the difference that the atmospheric conditions in the warm room were made such that the exposure of the upper three-fourths of the dog to this atmosphere prevented shivering. The results are illustrated in fig. 1 where the increase in the total blood solids from 13.2 to 15.4 per cent is seen to have occurred during the first ten minutes of exposure to a cold hip bath. The degree of cold anhydremia is somewhat proportional to the extent of the area exposed, as shown by the latter portion of the figure, the total solids undergoing a further increase of over 1 per cent as a result of immersion to the neck.

The above evidence indicates that a vital part in cold anhydremia and edema is played by direct peripheral action. It is true that dogs whose spinal cords have been severed at the level of the sixth cervical segment (Barbour and Tolstoi, 1924) do not respond to cold baths by any significant concentration of the blood. The pathology of these animals, however, is evidently complicated especially as regards their blood and lymph circulation, as shown, for example, by the permanent lowering of the total blood solids which the operation produces. This viewpoint was perhaps not given sufficient attention when the cord dog experiments were invoked to emphasize the control of water shifting by the central heat-regulating mechanism.

That the direct action of cold upon certain exposed areas of the body sets into play a local mechanism for the transference of fluid from blood to tissues does not of course militate in the slightest degree against accepting the fact that the body possesses a thermostat. The existence of such a central heat-regulating mechanism controlled by the blood temperature is of course shown in various ways by the experiments of Barbour (1912), Barbour and Wing (1913), Hashimoto (1915), Barbour and Prince (1914), Prince and Hahn (1918), Lillian Moore (1918), Sherrington (1924) and others.

Since the transfer of water which we have described evidently depends on peripheral vaso-constriction, one can not doubt that it can be controlled by the central heat-regulating mechanism through the vasomotor centers.

CONCLUSION

The following train of events accounts for the manner in which water leaves the blood of animals exposed to cold environments:

(1) Direct cooling of arterioles, or
(2) Cooling of cutaneous nerves → axone, spinal and prespinal reflexes

→ constriction of arterioles → slowing of blood flow → anoxemia → capillary relaxation → increased permeability (edema, anhydremia)

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THE BEGINNING OF ADRENAL FUNCTION IN THE EMBRYO CHICK

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Early writers have called attention to the relatively larger size of the adrenal gland in the embryo than in the adult (Soulié, 1903). In fact, Valenti (1889) has even considered the gland to be rudimentary in the adult partly on this basis. Fenger (1912) has shown that the active principle of the adrenal is present in the fetus of cattle, sheep and hogs at about the sixth week after conception. In a later paper (1912a) he has reported that the adrenals taken from cattle fetuses from six weeks to five months old and from suckling calves six weeks old yield a relatively larger amount of epinephrin than the adrenals of the adult. McCord (1915) found that the adrenals of cattle fetuses, at the end of the sixth week, are capable of relaxing the smooth muscle of guinea-pig uterus. Undoubtedly the adrenal hormone is present and capable of functioning at a very early age in the mammalian fetus.

Hogben and Crew (1923) have found the adrenal of chick embryos functional, by the intestinal strip method, at the sixteenth day of incubation in a few preliminary experiments. Several trials at the fourteenth day gave doubtful results.

The adrenal glands and the sympathetic nervous system are so closely related physiologically that one might expect these two systems to be correlated in functional development. This paper presents some observations on the beginning adrenal function in the embryo chick, and discusses them with reference to the development of the sympathetic system.

METHOD. The mydriatic action of adrenalin on the enucleated eye of the frog, first described by Meltzer and Auer (1904), was used as a basis for testing the embryonic adrenal. Ehrmann (1905) has described a method which permits a comparison of a control eye and a test eye at the same time. His method was followed in its essential details. Two cylindrical glass cells about 10 mm. deep and 18 mm. in diameter were cemented side by side to a glass microscope slide. The cells were partly filled with paraffin and a depression made in the center of each paraffin layer to hold the eye with the pupil uppermost. The long or horizontal diameter and the short or vertical diameter could then be measured by means of an ocular micrometer.

The two eyes of the frog (Rana pipiens) were excised after the brain had been pithed and the dorsal part of the head had been cut off at the anterior border of the ear drums. Each eye was put in a glass cell containing about 1 cc. of Ringer's solution for cold-blooded animals, and then covered with a cover slip. Three sets of preliminary readings were usually taken on each eye at intervals of ten minutes.

The adrenals of the embryo chick were removed under a binocular microscope. All adherent tissue was removed and the glands were extracted in a mortar with about 1 cc. of Ringer's solution. This was not done until the preliminary readings of both pupils in Ringer's solution showed that a definite constriction was taking place. The adrenal extract was always used immediately. The Ringer's solution was then removed from the test eye and the adrenal extract substituted for it. The Ringer's solution of the control eye was changed at the same time in order to control possible mechanical effects. Measurements of the diameters of the pupils were then made about every ten minutes until the end of the experiment.

The use of a control eye subjected to the same external conditions served to control any lighting, mechanical or temperature effects. However, the lighting conditions were kept as constant as possible. Since we were working with a dilatation, it was thought desirable to be sure that a constriction was starting in both pupils, and for this the general lighting of the room was usually sufficient. In order to facilitate reading the micrometer a 50 watt daylight lamp set fourteen inches above and ten inches in front of the stage was turned on for a brief period during the actual measurement. An inhibition of the constriction in the test eye, or a dilatation would then be significant if a constriction were going on in the control.

Additional controls were carried out by using various other tissues, such as gonad, mesonephros, metanephros, heart muscle, liver, spleen and cerebrum in place of the adrenal gland. The customary procedure was to use two pairs of eyes and to test the adrenal glands and some other tissue from the same embryo each with its Ringer control.

Results. The adrenals of forty-two embryo chicks of various ages between seven and twenty days were tested. Twenty-seven were positive for an active dilating principle, ten were negative, and five were doubtful. All the doubtful and negative cases were in chicks from seven to ten days old, although one positive test was obtained from a chick of eight days, four from nine-day chicks, and five from ten-day chicks. All tissue controls, thirty in number, were negative. Seventy-two Ringer controls showed constriction of the pupil in fifty-five cases, dilatation in nine cases, and no change in size in eight cases.

The pH of the extracts of adrenal, mesonephros, gonad, liver, heart

TABLE 1

The mydriatic action of extract of the adrenals of chicks from eight to sixteen days of incubation, and Ringer controls

REMARKS	TIME	CONTROL PUPIL DIAMETERS		TEST PUPIL DIAMETERS	
		Horizontal	Vertical	Horizontal	Vertical
		mm.	*mm.*	*mm.*	*mm.*
Experiment 5. Age 16 days. Adrenal extract added at 4:46	4:10	2.60	2.10	2.85	2.25
	4:30	2.35	1.60	2.45	1.60
	4:45	2.15	1.30	2.25	1.25
	4:46				
	5:00	2.20	1.25	2.30	1.25
	5:15	2.30	1.40	2.55	2.30
	5:25	2.25	1.40	2.65	2.65
	5:35	2.25	1.35	2.75	2.75
	5:45	2.15	1.30	2.75	2.80
	6:00	2.15	1.25	2.75	2.90
Experiment 17. Age 14 days. Adrenal extract added at 4:02	3:15	2.60	1.90	2.25	1.50
	3:25	2.30	1.55	2.05	1.30
	4:00	2.00	1.20	2.00	1.05
	4:02				
	4:15	1.90	1.15	2.10	1.20
	4:30	1.85	1.15	2.35	1.40
	4:40	1.95	1.10	2.35	1.40
	4:50	2.05	1.20	2.50	1.65
Experiment 15. Age 13 days. Adrenal extract added at 4:32	3:45	2.45	1.70	2.60	1.85
	4:05	2.10	1.20	2.15	1.20
	4:30	1.90	0.90	1.90	0.85
	4:32				
	4:40	1.80	0.90	1.90	0.85
	4:55	1.90	0.95	2.25	1.25
	5.05	1.80	0.90	2.35	1.30
	5:15	1.80	0.85	2.35	1.25
	5:25	1.75	0.95	2.40	1.35
	5:35	1.75	0.85	2.40	1.30
	5:55	1.75	0.85	2.50	1.45
Experiment 13. Age 11 days. Adrenal extract added at 4:42	4:15	2.40	1.70	2.35	1.55
	4:20	2.20	1.40	2.25	1.30
	4:40	1.80	0.60	1.70	0.65
	4:42				
	4:55	1.60	0.70	1.75	0.70
	5:10	1.65	0.60	1.95	0.85
	5:20	1.55	0.60	2.05	0.90
	5:30	1.55	0.55	2.10	0.95
	5:40	1.55	0.60	2.10	0.95

TABLE 1—*Concluded*

REMARKS	TIME	CONTROL PUPIL DIAMETERS		TEST PUPIL DIAMETERS	
		Horizontal	Vertical	Horizontal	Vertical
		mm.	*mm.*	*mm.*	*mm.*
Experiment 24. Age 10 days. Adrenal extract added at 4:51	4:20	2.75	2.05	2.40	1.50
	4.40	2.75	1.75	2.30	1.15
	4:50	2.60	1.65	2.15	1.20
	4:51				
	5:20	2.25	1.45	2.50	1.55
	5:30	2.25	1.35	2.50	1.55
	5:50	2.10	1.10	2.50	1.45
Experiment 35. Age 9 days. Adrenal extract added at 9:42	9:25	3.15	3.05	3.00	2.50
	9:40	3.00	2.60	2.80	2.00
	9:42				
	9:50	2.80	2.35	2.65	1.85
	10:05	2.60	2.10	2.75	1.90
	10:15	2.45	1.85	2.80	2.00
	10:25	2.50	1.95	2.90	2.00
	10:35	2.40	1.80	2.90	2.00
	10:50	2.35	1.65	2.95	1.95
	11:00	2.20	1.35	3.00	1.95
	11:15	2.15	1.15	3.00	2.00
Experiment 46. Age 8 days. Adrenal extract added at 3:46	3:40	2.90	2.15	2.70	1.80
	3:45	2.80	1.90	2.65	1.65
	3:46				
	4:05	2.50	1.60	2.60	1.60
	4:15	2.40	1.40	2.60	1.55
	4:30	2.35	1.25	2.60	1.50
	4:40	2.30	1.15	2.60	1.45
	4:50	2.25	1.10	2.60	1.45
	5:00	2.30	1.10	2.65	1.50
	5:25	2.20	1.05	2.65	1.60
	6:00	2.20	1.10	2.90	1.90

muscle and spleen was determined by the colorimetric method of Clark and Lubs and was found to vary between 7.4 and 7.6. The pH of the control Ringer solution was 8.0. Since no tissue except adrenal showed any effect on the pupil, we are sure that this difference in pH is not responsible for the dilatation concerned. In many cases, however, the pH of the control Ringer was brought to 7.5 by HCl. No variations from the usual results could be detected.

Table 1 shows the protocols of seven experiments in which the presence of the active principle was indicated. The complete data showed that the dilatation always occurred in the positive cases within the first half-hour, but usually within the first fifteen minutes.

TABLE 2

The absence of mydriatic action of extracts of various tissues of embryo chicks, and Ringer controls

REMARKS	TIME	CONTROL PUPIL DIAMETERS		TEST PUPIL DIAMETERS	
		Horizontal	Vertical	Horizontal	Vertical
		mm.	*mm.*	*mm.*	*mm.*
	10:00	2.45	1.85	2.45	1.80
	10:25	2.30	1.55	2.25	1.45
	10:40	2.15	1.25	2.20	1.30
	10:41				
Experiment 12. Gonad, male. Age 14 days	10:50	2.10	1.25	2.10	1.20
	11:05	2.10	1.15	2.00	1.15
	11:20	2.05	1.05	1.95	1.05
	11:35	2.00	1.00	2.00	1.00
	11.45	2.10	1.00	2.15	1.00
	12:20	2.25	1.10	2.15	1.05
	4:10	2.45	1.50	2.60	1.65
	4:20	2.40	1.30	2.45	1.45
	4:45	2.15	1.15	2.20	1.10
	4:46				
Experiment 14. Mesonephros. Age 11 days. See experiment 13 for adrenal of same chick	4:55	1.95	0.95	2.05	0.95
	5:05	1.95	0.95	2.10	1.10
	5:20	2.00	1.00	2.10	1.10
	5:30	2.00	1.00	2.10	1.10
	5:40	2.00	0.95	2.05	1.10
	5:50	1.95	1.00	2.00	1.00
	3:45	2.30	1.50	2.35	1.55
	4:05	1.90	1.00	2.05	1.10
	4:40	1.50	0.65	1.65	0.75
	4:42				
Experiment 16. Metanephros. Age 13 days. See experiment 15 for adrenal of same chick	4:50	1.45	0.60	1.60	0.75
	5:05	1.45	0.60	1.60	0.70
	5:15	1.55	0.70	1.70	0.80
	5:25	1.50	0.60	1.60	0.75
	5:35	1.50	0.65	1.70	0.80
	4:05	3.10	2.35	3.00	2.20
	4:15	3.60	3.25	3.20	2.90
	4:30	3.45	2.85	3.10	2.55
	4:31				
Experiment 49. Liver. Age 8 days	4:45	3.05	2.60	2.90	2.10
	4:50	2.90	2.45	2.65	1.90
	5:15	2.45	1.85	2.50	1.50
	6:00	2.00	1.30	2.25	1.25

TABLE 2—*Concluded*

REMARKS	TIME	CONTROL PUPIL DIAMETERS		TEST PUPIL DIAMETERS	
		Horizontal	Vertical	Horizontal	Vertical
		mm.	*mm.*	*mm.*	*mm.*
Experiment 53. Heart muscle. Age 8 days	9:25	1.80	1.20	1.90	1.00
	9:55	1.75	0.90	1.80	0.90
	9:57				
	10:20	1.55	0.85	1.65	0.75
	10:40	1.55	0.80	1.65	0.75
	11:00	1.60	0.75	1.60	0.70
	11:20	1.55	0.75	1.55	0.75
Experiment 62. Spleen. Age 11 days	3:35	3.45	2.75	3.45	3.05
	3:40	3.20	2.35	3.10	2.20
	3:52				
	4:00	2.95	1.95	2.95	1.90
	4:15	2.80	1.70	2.80	1.70
	4:30	2.60	1.40	2.60	1.45
	5:00	2.45	1.25	2.45	1.25
Experiment 70. Cerebrum. Age 16 days	2:45	2.40	1.35	1.95	1.20
	3:10	2.25	1.10	2.05	1.00
	3:12				
	3:20	2.15	1.05	2.00	1.05
	3:30	2.05	1.05	1.85	0.95
	4:00	2.10	1.05	1.75	0.80

Table 2 shows the protocols of seven experiments with indifferent tissues including mesonephros, metanephros, gonad, liver, spleen, heart muscle and cerebrum. The absence of any effect on the pupil by the liver extract confirms the observation of Cannon and Uridil (1921) that the liver hormone, for the existence of which they present evidence, has no influence on the sensitized iris.

Figure 1 shows the effect of the adrenals of an eleven-day embryo chick. Liver and spleen of the same chick were also tested. All three tissues had Ringer controls. Only the pupil in the adrenal extract showed a dilatation.

DISCUSSION. Negative results may be due to the absence of the hormone, to a high threshold in the eyes used, or to failure to obtain and extract the adrenal in the very young stages. The earliest age at which the extract of the adrenal would give a positive test on the pupil, provided the active principle were present, would be determined by the threshold of the pupil. Shultz (1909) found the pupil sensitive to solutions of 1:3,125,000 in some instances, but only solutions of 1:125,000 or stronger gave uniform results, while those which were weaker gave very uncertain

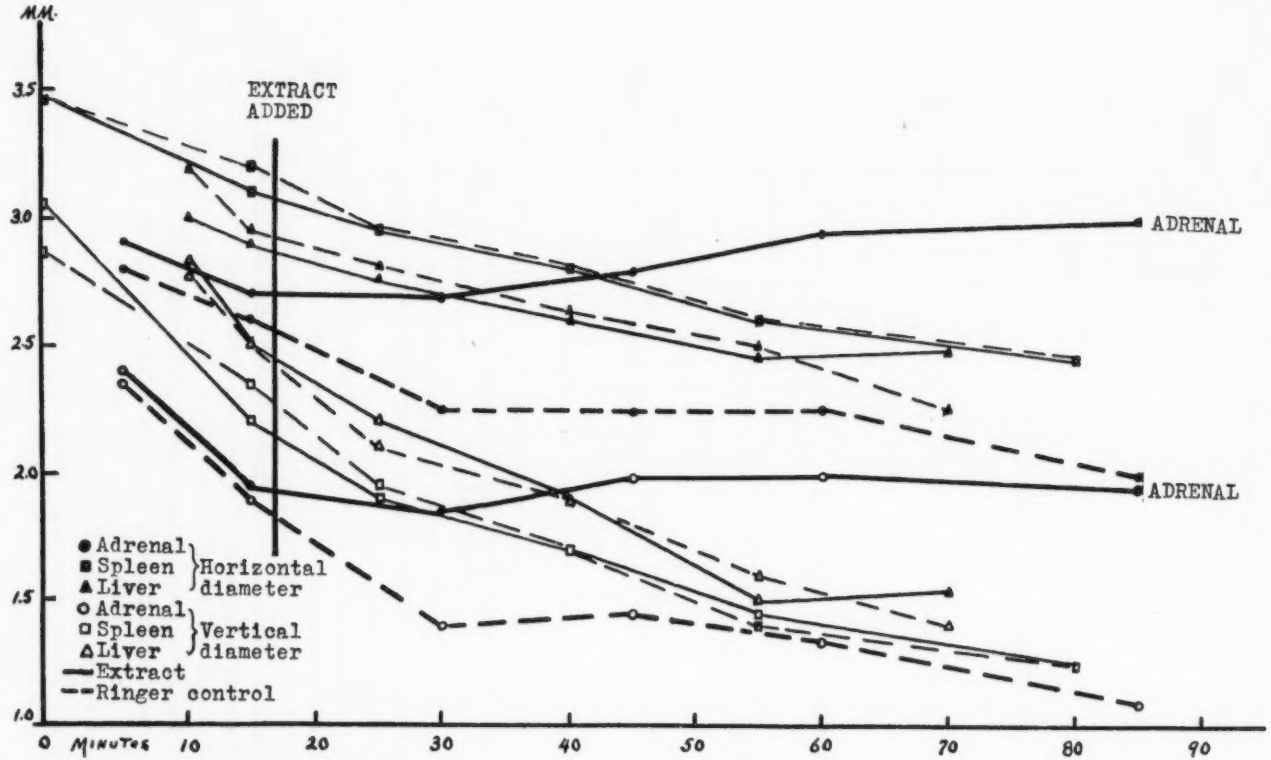

Fig. 1. The mydriatic effect of Ringer extract of the adrenals of a chick eleven days after the beginning of incubation. The absence of this effect by extracts of the liver and spleen of the same chick is also shown. Circles denote the adrenal test; squares the spleen; triangles the liver. Solid lines indicate extracts of tissue, dotted lines, Ringer controls. Solid characters represent horizontal diameters, hollow characters vertical diameters. The time of addition of the extract is denoted by the vertical line seventeen minutes after the start.

results. We found solutions of adrenalin chloride, 1:500,000, to be without effect in some cases. The difficulties in removing and extracting the minute adrenal in eight- to ten-day embryo chicks are many. The tissue is soft, watery and jelly-like, and the mass of tissue obtained is sometimes hardly more than 1 mm. in diameter.

From a consideration of the embryological development of the adrenal as correlated with our present physiological findings we are inclined to believe that the adrenal is not sufficiently developed to function earlier than the eighth day of incubation. Lillie (1919) pointed out that the ingrowth of the medullary cords from the large sympathetic ganglion lying on the dorso-medial surface of the cortical mass does not begin until the eighth day. By the eleventh day they have penetrated about a third of the thickness of the gland. The cortical cords are well developed at the eighth day. Thus, if we found the adrenal functional earlier than the eighth day, it would suggest that the medullary cells contained the hormone. There is some suggestive evidence for each view. Gaskell (1919) reported the finding of an adrenalin-like substance in the nerve ganglia of leeches. Hartman (1923) has presented evidence that the cortical cells of the adult gland produce adrenalin.

The sympathetic nervous system and the adrenalin secreting system are in every vertebrate most intimately connected physiologically and anatomically. In the chick the ganglia of the sympathetic nervous system are well developed at the eighth day of incubation, according to Lillie (1919), but no literature was found on the time of association of the post-ganglionic fibers with the smooth muscle of the walls of the blood vessels. Kuntz (1910) found no traces of the anlagen of the secondary sympathetic trunks in the posterior part of the body until the close of the sixth day, but during the seventh and eighth days of incubation the sympathetic plexuses in the walls of the digestive tube become well established. We should hardly expect, therefore, the innervation of the blood vessels to take place earlier than the eighth day. Probably it occurs later.

Gaskell (1920) pointed out that in the lowest vertebrates the function of the sympathetic nervous system is largely taken over by a chromaffine system. This early phylogenetic functioning of the chromaffine system supports the idea that in the embryos of higher vertebrates the adrenals may function before the sympathetic nervous system. Certainly the observations of Fenger (1912a) suggest that they have a relatively more important rôle in the embryo than in the adult. More detailed information concerning the later embryological development of the sympathetic nervous system of the blood vessels is needed.

SUMMARY

1. The adrenal glands of forty-two embryo chicks at various ages between seven and twenty days were extracted in Ringer's solution and the mydriatic action determined on the enucleated eye of the frog. Twenty-seven cases were positive, ten cases were negative, and five cases were doubtful.

2. All negative and doubtful cases were in chicks from seven to ten days of incubation, but positive evidence of an active agent was found as early as the eighth day, and also during the ninth and tenth days.

3. Thirty control tests were carried out on other tissues including mesonephros, metanephros, gonad, liver, spleen, heart muscle and cerebrum. No evidence of a dilating agent was found.

4. The functional relationship of the development of the adrenals and the sympathetic nervous system is discussed.

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